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# **Characterisation Of Aphid Proteins As Targets For Aphid Control.**

A thesis submitted for the Degree of Doctor of Philosophy by

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June 1998

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	<b>Contents</b>	<b>Page</b>
	Abstract	vi
	Acknowledgements	viii
	Declaration	ix
	List of Figures	x
	List of Tables	xii
	List of Abbreviations	xiii
<b>1</b>	<b>Introduction</b>	<b>1</b>
<b>1.1</b>	<b>Invertebrate Pests</b>	<b>1</b>
1.2.1	Aphid Pests	4
1.2.2	Aphid Life-cycle	5
<b>1.3</b>	<b>Current Methods of Invertebrate Control</b>	<b>7</b>
1.3.1.1	<u>Insecticides</u>	7
1.3.1.2	<u>Insecticide Resistance</u>	7
1.3.2	Tolerance To Insect Pests	11
1.3.3	Avoidance of Insect Pests	12
1.3.4	Plant Resistance to Insect Pests	12
1.3.4.1	<u>Physical Characteristics</u>	13
1.3.4.2	<u>Plant Surface Chemicals</u>	13
1.3.4.3	<u>Internal Plant Biochemistry</u>	13
<b>1.4</b>	<b>Enhancement of Plant Resistance To Pests</b>	<b>14</b>
<b>1.5</b>	<b>Pest Responses To Plant Resistance</b>	<b>16</b>
<b>1.6</b>	<b>A Novel Target For Aphid Control</b>	<b>18</b>
<b>1.7</b>	<b>Analysis of Aphid Gut Proteins</b>	<b>23</b>
1.7.1	Gel Electrophoresis	23
1.7.2	Enzyme Electrophoresis	25
1.7.3	Two Dimensional Polyacrylamide Gel Electrophoresis (2DE)	26

<b>1.8</b>	<b>Availability of Screening Material</b>	<b>27</b>
<b>1.9</b>	<b>Research Aims</b>	<b>28</b>
<b>1.10</b>	<b>Plan of Work</b>	<b>31</b>
<b>2</b>	<b>Materials and Methods</b>	<b>32</b>
<b>2.1</b>	<b>Materials</b>	<b>32</b>
2.1.1	Chemicals	32
2.1.2	Plants	32
2.1.3	Aphids	32
2.1.3.1	<u>Stock Aphid Cultures</u>	32
2.1.3.2	<u>Field Collected Aphids</u>	33
<b>2.2</b>	<b>Methods</b>	<b>33</b>
2.2.1	Preparation of aphid samples for gel electrophoresis and antibody production	33
2.2.1.1	<u>Dissection of aphid guts and remnants</u>	33
2.2.1.2	<u>Detergent extracts of aphid tissues</u>	34
2.2.2	Determination of protein concentration	35
2.2.3	Polyclonal antiserum production	35
2.2.3.1	<u>Preparation of whole insect immunogen</u>	35
2.2.3.2	<u>Preparation of aphid gut immunogen</u>	36
2.2.3.3	<u>Immunisation protocol</u>	36
2.2.4	Artificial diet	36
2.2.4.1	<u>Aphid Diet Solutions</u>	38
1	Solution 1: [100x] vitamin solution	38
2	Solution 2: [1000x] solution of trace elements	38
3	Solution 3: [2x] amino acid and mineral solution	39
4	Solution 4: tyrosine solution	39
2.2.5	Metabolic labelling of aphid gut proteins	40
2.2.6	Isolation of Immunoglobulin G (IgG) from aphid tissues	40

2.2.7	Analysis of Aphid Proteins	41
2.2.7.1	<u>One dimensional polyacrylamide gel electrophoresis (1DE)</u>	41
2.2.7.2	<u>Two dimensional electrophoresis (2DE)</u>	42
1	Sample preparation	42
2	First Dimension - IEF gel electrophoresis	42
3	Second Dimension - SDS PAGE	43
2.2.7.3	<u>Silver staining</u>	43
2.2.7.4	<u>Western blotting and protein detection with antibody</u>	44
2.2.7.5	<u>Western blotting and protein detection with biotinylated lectins</u>	45
2.2.8	Analysis of protein spots on 2DE	46
<b>3</b>	<b>Tissue specific protein variation between aphid species</b>	48
<b>3.1</b>	<b>Introduction</b>	48
<b>3.2</b>	<b>Results</b>	50
3.2.1	One Dimensional Electrophoresis	50
3.2.2	Metabolic labelling of aphid proteins	52
3.2.3	2DE	54
3.2.3.1	<u>Remnant Proteins</u>	54
3.2.3.2	<u>Gut Proteins</u>	61
3.2.4	Protein Identification	67
<b>3.3</b>	<b>Discussion</b>	68
<b>4</b>	<b>Host Plant Induced Changes In Aphid Gut Proteins</b>	72
<b>4.1</b>	<b>Introduction</b>	72
<b>4.2</b>	<b>Results</b>	73
4.2.1	One Dimensional Electrophoresis of gut proteins from <i>M. persicae</i> on different crop plants	73

4.2.2	One Dimensional Electrophoresis of gut proteins from <i>M. persicae</i> and <i>Ma. euphorbiae</i> fed on different crop plants	75
4.2.3	Two Dimensional Electrophoresis & Silver Staining	78
4.2.4	2DE and Western Blotting	81
4.2.4.1	<u>2DE Blots Of Gut Protein Probed With Peanut Agglutinin (PNA)</u>	81
4.2.4.2	<u>2DE Blots Of Gut Protein Probed With Concanavalin A (Con A)</u>	83
4.2.4.3	<u>2DE Blots Of Gut Protein Probed With Antibody</u>	85
4.3	<b>Discussion</b>	87
<b>5</b>	<b>Characterisation of antisera raised against aphid proteins</b>	91
5.1	<b>Introduction</b>	91
5.2	<b>Results</b>	92
5.2.1	Production of Antibodies to <i>M. persicae</i> Proteins	92
5.2.1.1	<u>Anti-Whole <i>M. persicae</i> Antibody</u>	92
5.2.1.2	<u>Anti-<i>M. persicae</i> Gut Antibody</u>	92
5.2.3	Anti-Whole <i>M. persicae</i> Antibody Characterisation	94
5.2.4	Anti- <i>M. persicae</i> Gut Antibody Characterisation	96
5.2.4.1	<u>Collection of anti- <i>M. persicae</i> gut antibody</u>	96
5.2.4.2	<u>Anti- <i>M. persicae</i> gut antibody binding to proteins from different aphid species using 1DE</u>	98
5.2.4.3	<u>Anti- <i>M. persicae</i> gut antibody binding to proteins from <i>M. persicae</i> on 2DE</u>	100
5.2.4.4	<u>Anti- <i>M. persicae</i> gut antibody binding to 2DE separated proteins from a <i>M. persicae</i> clone fed on two different host plants</u>	100
5.3	<b>Discussion</b>	102

<b>6</b>	<b>Effects of anti-<i>M. persicae</i> antibodies on feeding aphids</b>	<b>107</b>
<b>6.1</b>	<b>Introduction</b>	<b>107</b>
<b>6.2</b>	<b>Methods</b>	<b>108</b>
<b>6.3</b>	<b>Results</b>	<b>109</b>
6.3.1	Anti-whole <i>M. persicae</i> antibody	109
6.3.2	Anti- <i>M. persicae</i> gut antibody	111
6.3.2.1	<u>Parameters used to assess effect of treatment</u>	111
6.3.2.2	<u>Effects of antiserum concentration on feeding aphids</u>	111
6.3.2.3	<u>Objectivity in results of feeding trials</u>	113
6.3.3	Movement of serum components from artificial diet into the aphids	115
<b>6.4</b>	<b>Discussion</b>	<b>119</b>
<b>7</b>	<b>Final Discussion</b>	<b>122</b>
<b>7.1</b>	<b>The Variation Of Aphid Proteins Between Species</b>	<b>122</b>
<b>7.2</b>	<b>Induced Differences In Aphid Gut Proteins</b>	<b>126</b>
<b>7.3</b>	<b>Implications Of The Variation In Aphid Gut Protein</b>	<b>129</b>
<b>7.4</b>	<b>A Novel Target For Aphid Control</b>	<b>132</b>
<b>7.5</b>	<b>Conclusions</b>	<b>136</b>
	<b>References</b>	<b>137</b>
	<b>Publications And Abstracts Of Scientific Meetings</b>	<b>162</b>
	<b>Appendix 1</b>	<b>163</b>

## Abstract

There have been extensive investigations of allozymes in aphid species, but only a relatively small amount of variation has been found between and, especially, within species. While modern molecular methods have shown that there is a large amount of variation amongst and within aphid species at the DNA level, there has been no concomitant detailed investigation of protein variation.

2DE was used as a powerful method to analyse the protein complement of aphid tissues. When the protein profiles of remnant and gut tissues were compared within and between several aphid species, higher levels of conservation were found in remnant tissue proteins than in the gut tissue proteins. These conservation levels may indicate different evolutionary processes in the two tissue types. The remnant proteins may have specific functions across all aphid species, which restrict the chances of accumulating mutations. The gut proteins do not appear to be similarly constrained, with the wide variation in gut protein profiles observed amongst aphid species possibly related to differences in their host ranges. The presence of protein homologues or common precursor molecules was indicated where some protein appeared to have slight, but distinct, differences between the species.

The protein data from both tissues was qualitatively analysed to produce parsimonious comparisons between the aphid species. The gut protein data gave strong relationships between the species, which were in agreement with a classification based on aphid morphometrics. However, the high level of conservation in the remnant proteins appeared to have obscured any separation of the species using this data.

The effects of changing diet on the proteins of the aphid gut were also explored using 2DE. Within each clone, and therefore within each species, a small subset of proteins varied with host plant. On both host plant species, an analysis of this variation found that the changes included both additions and absences within the aphid gut protein profile.

A polyclonal antiserum was raised against total proteins from *M. persicae*, fed on Chinese cabbage. The cross-reactivity of anti- whole *M. persicae* antiserum with large numbers of Western blotted proteins from other aphid species confirmed the protein conservation observed after 2DE protein analysis.

A second polyclonal antiserum, raised against gut proteins from *M. persicae* fed on Chinese cabbage, also showed cross-reactivity with Western blotted proteins from other aphid species. Probing with lectins, which specifically bind to secondary carbohydrate structures, showed that many of these cross-reacting gut proteins were glycosylated. As has been found with some antisera raised against proteins from other insects, the secondary carbohydrate structure of the proteins may account for some of the cross-reactivity seen with proteins from other species. The cross-reactivity of the anti-gut antibody may also indicate the presence of homologous proteins occurring in the guts of aphid species, previously indicated after 2DE separation and silver staining of aphid proteins.

After establishing a suitable artificial diet for the long term culture of *M. persicae*, the effects of including the polyclonal antisera raised against aphid proteins in the diet were assessed. Inclusion of anti- *M. persicae* gut antiserum in artificial diet had a detrimental effect on the longevity of feeding aphids.

The findings of the thesis are discussed in context of aphid control and the current trend towards *in planta* methods.

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Finally, I want to thank my folks, without whom I would not be the person that I am today.

"..Ignotus pecori.."

Catullus, 87-54? B.C.



**Declaration**

I hereby declare that this thesis is based on the results of experiments done by myself and that it is of my own composition. This thesis has not, in whole or in part, been presented for a higher degree or qualification elsewhere.

The research was carried out at the Scottish Crop Research Institute, Invergowrie, Dundee, under the supervision of Dr Brian Fenton.

Figures	Figure Title	Page
Figure 1.1	General aphid life-cycle showing host alteration and changes in reproduction with season	6
Figure 1.2	The immunity and cross-immunity of guinea pigs to three tick species following immunisation with antigens from each of the ticks	22
Figure 3.1	Differences in protein profiles between aphid species after separation using 1DE	49
Figure 3.2	Metabolic labelling of aphid proteins from two aphid species	51
Figure 3.3	2DE separation of remnant proteins from three aphid species	53
Figure 3.4	Composite map of remnant proteins from five aphid species	55
Figure 3.5	Dendrograms produced following Dollo and Wagner parsimony of remnant protein data	58
Figure 3.6	Gut proteins from three aphid species separated using 2DE	60
Figure 3.7	Composite map of gut proteins from six aphid species	62
Figure 3.8	Identification of low molecular weight proteins unique to different aphid species	65
Figure 3.9	Dendrograms produced following Dollo and Wagner parsimony of gut protein data	66
Figure 4.1	1DE comparison of gut proteins from <i>M. persicae</i> collected from different host plants	74
Figure 4.2	1DE analysis of gut proteins from two aphid species feeding on two different host plants	76
Figure 4.3	2DE analysis of gut proteins from <i>M. persicae</i> feeding on two different host plants	79
Figure 4.4	Pattern of PNA binding to gut proteins from <i>M. persicae</i> feeding on two different host plants	82

Figure 4.5	Pattern of Con A binding to gut proteins from <i>M. persicae</i> feeding on two different host plants	84
Figure 4.6	Pattern of anti- <i>M. persicae</i> gut antibody binding to gut proteins from a <i>M. persicae</i> clone fed on two different host plants	86
Figure 5.1	Detergent extracts of aphid gut proteins	93
Figure 5.2	Binding patterns of anti-whole <i>M. persicae</i> antibodies to proteins from four aphid species	95
Figure 5.3	Binding patterns of anti- <i>M. persicae</i> gut antibodies to proteins from four aphid species	97
Figure 5.4	Comparison of total gut proteins and binding of anti- <i>M. persicae</i> gut antibodies to <i>M. persicae</i> gut proteins	99
Figure 5.5	Binding of anti- <i>M. persicae</i> gut antibodies to gut proteins from a <i>M. persicae</i> clone fed on two different host plants	101
Figure 6.1	Effects of anti-whole <i>M. persicae</i> antibodies on feeding aphids	110
Figure 6.2	Effects of three concentrations of anti- <i>M. persicae</i> gut antibodies on feeding aphids	112
Figure 6.3	The effects of anti- <i>M. persicae</i> gut antibodies (1:50) on feeding <i>M. persicae</i>	114
Figure 6.4	Inter-sachet variation observed within treatments over one feeding trial	116
Figure 6.5	Passage of IgG into aphids from AD	118

<b>Tables</b>	<b>Table Title</b>	<b>Page</b>
Table 1.1	Some crops and their major pests	1
Table 1.2	Some insect vectors and examples of the viruses that they transmit	3
Table 2.1	Artificial diet. Solution 1: [100x] vitamin solution	38
Table 2.2	Artificial diet. Solution 2: [1000x] trace element solution	38
Table 2.3	Artificial diet. Solution 3: [2x] amino acid and mineral solution	39
Table 3.1	Remnant proteins present in the protein profiles of five aphid species	56
Table 3.2	Proteins found to be unique to individual aphid species	57
Table 3.3	Gut proteins present in the protein profiles of six aphid species	63

## Abbreviations

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AD	artificial diet
A.i.	<i>Amphorophora idaei</i>
A.s.	<i>Aulacorthum solani</i>
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLRV	pea (or bean) leaf roll virus
BMV	beet mild yellows virus
BSA	bovine serum albumin
BYNV	beet yellow net virus
Con A	concanavalin A
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetic acid
Fab	antibody fragment with antigen binding site
<i>g</i>	units of gravitational force
IEF	isoelectric focusing
IgG	immunoglobulin G
kDa	kilo Daltons
M	molar
M.a.	<i>Myzus antirrhinii</i>
MACE	modified acetylcholine esterase
M.e.	<i>Macrosiphum euphorbiae</i>
M.p.	<i>Myzus persicae</i>
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
MW	standard molecular weight markers
NBT	4-nitroblue tetrazolium chloride
nm	nano metres (wavelength)
NP40	nonidet P40

NSS	normal sheep serum
PA	protective antigen
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEMV	pea enation mosaic virus
pers comm	personal communication
pI	isoelectric point
PI	protease inhibitor
PLRV	potato leaf roll virus
PM	peritrophic membrane
PNA	peanut agglutinin
PVX	potato virus X
PVY	potato virus Y
P1	pellet 1
RAPDs	random amplified polymorphic DNA
rDNA	ribosomal DNA
RNA	ribonucleic acid
R.p.	<i>Rhopalosiphum padi</i>
SCRI	Scottish Crop Research Institute
SDS	sodium dodecyl sulphate
sp.	species
S1	supernatant 1
S2	supernatant 2
S3	supernatant 3
S4	supernatant 4
S5	supernatant 5
TEMED	N,N,N <sup>1</sup> ,N <sup>1</sup> -tetramethylethylenediamine
TET	Tris-EDTA-Triton X-100 buffer
TNTT	Tris-sodium chloride-thimerosal-tween buffer

Tris	tris(hydroxymethyl)amino methane
TVNV	tobacco veinal necrosis virus
v/v	volume per volume
w/v	weight per volume
1DE	one-dimensional electrophoresis
2DE	two-dimensional electrophoresis
$\alpha$	anti (denoting antibody)

## Chapter 1

### Introduction

#### 1.1 Invertebrate Pests

Agricultural and horticultural pests can be defined as those organisms which, by feeding or other action, cause damage or detrimental changes to their hosts. While there are mammals and birds which damage crops, the majority of crop pests are invertebrates. This study concentrates on aphid pests, but important parallels can be drawn with other pests of plants and parasites of vertebrates. All crops have at least one pest and some examples of crops with their associated pests are given in Table 1.1.

Crop	Major pest species
wheat	aphids, hessian fly, wheat mite
rice	planthoppers, plant borers, gall midge
maize	corn borers, rootworms, army worm, stalk borers
potato	aphids, colorado potato beetle, potato leaf hopper, wireworms
rape	aphids, brassica pod midge, flea beetles, cabbage stem & seed weevils
sugar beet	leaf hoppers, aphids, mangold fly
grapes	grape leafhopper, grape phyloxera, mealybugs
tomato	whitefly, thrips, green stinkbug, leafminers, fruitworm
oats	aphids, frit fly
rye	aphids, frit fly
raspberry	raspberry beetle, large raspberry aphid, cane midge
Ribes sp.	gall midge, cane midge, sawfly
strawberry	clay coloured weevil, strawberry aphid

**Table 1.1**

**Some crops and their major pests** (adapted from Hill, D.S., 1987).



Damage and yield reduction by pests occurs throughout the world, with an average loss of between 10-20 % per year for all crops (Oerke *et al.*, 1994). Approximately 16 % of the total production of the eight principal food and cash crops (wheat, rice, coffee, cotton, potatoes, soybean, barley, and maize) was lost to pests during the 1988-1990 growing seasons (Oerke *et al.*, 1994). The losses to these crops occurred even though control methods, mainly insecticides, were used.

Many invertebrates are pests due to their ability to rapidly increase in numbers and cause direct feeding damage. For example, initially small populations of locusts can be stimulated by external cues, such as temperature fluctuation, to produce very large numbers of offspring (Risch, 1987). As locusts are very mobile and able to feed on many plant species, i.e. they are highly polyphagous, sudden increases in locust numbers can lead to very high levels of damage to many crops (Nevo, 1996).

Aside from direct feeding damage, many organisms are plant pests because of their ability to transmit harmful viruses (Table 1.2). There are over 400 known animal vectors of plant viruses; approximately 94 % of these are insects, 5 % are nematodes and less than 1 % are other animals and birds (Harris, 1981; Brunt *et al.*, 1996). Insects with piercing or sucking mouthparts constitute the majority of invertebrate virus vectors (Harris, 1981) and 76 % of these are members of the order Homoptera, suborder Sternorrhyncha (Harris 1982).

Viruses can cause significant reductions in crop yields (Matthews, 1991), but where vectoring is essential for transmission, control can be achieved by targeting the virus vector. For example, potato virus Y is spread by aphids, and effective aphid control in South African pepper crops led to a 32 % yield increase over controls (Budnik *et al.*, 1996). Also, the incidence of wheat streak mosaic virus was reduced by

Order	Family	Common name	Examples of viruses transmitted
	Aleyrodidae	whitefly	gemini viruses & leaf curls
	Aphididae	aphids	> 164 viruses, inc. luteoviruses & potyviruses
<b>Homoptera</b>	Cicadellidae	leafhoppers	rice dwarf, maize streak, potato yellow dwarf
	Delphacidae	planthoppers	maize rough dwarf, rice grassy stunt
	Membracidae	treehoppers	tomato pseudo curly top
	Psuedococcidae	mealybugs	cacao mottle leaf, dasheen mosaic
	Lygaeidae	plant bugs	centrosema mosaic
<b>Hemiptera</b>	Miridae	tarnished plant bugs	spinach blight
	Piesmidae	lace bugs	beet savoy, beet leaf curl
<b>Thysanoptera</b>	Thripidae	thrips	tomato spotted wilt
	Chrysomelidae	leaf beetles	broad bean mottle, southern bean mosaic
<b>Coleoptera</b>	Coccinellidae	ladybirds	squash mosaic, bean yellow stipple
	Curculionidae	snout weevils/beetles	broad bean stain, scrophularia mottle
	Meloidae	blister beetles	bean pod mottle
<b>Diptera</b>	Agromyzidae	leafminers	celery mosaic, tobacco mosaic

**Table 1.2**

**Some insect vectors and examples of the viruses that they transmit**

(adapted from Harris, 1982).

an average of 76 % when the mite vector *Eriophyes tulipae* (Kieffer) was controlled (Harvey *et al.*, 1994).

Many invertebrates have become pests by the intervention of man. This has occurred either by removal of predators, moving the pests into new environments where predation of the pest is reduced or absent, or introducing suitable host plants into the established range of a pest. Modern agricultural practices have also created new opportunities for insects to attack crops. Over the past 100 years there has been a shift in agrosystems towards the planting of crops which have a reduced genetic diversity, often down to the level of monocultures (Risch, 1987), so at any given time there is increased uniformity of plant age and physical quality within a crop. For insect pests, this trend towards crop genetic uniformity reduces the amount of energy that has to be

expended in finding a suitable host, and allows large numbers of pests to build up (Wilhoit, 1992).

### 1.2.1 Aphid Pests

Aphids are one of the most widespread groups of insect plant pests. They feed on the nutritionally poor phloem sap and to survive they must consume large volumes (Srivistava, 1987). Feeding requires probing the plant surface with the stylet until the phloem tube is located and then penetrated to allow removal of sap (Klingauf, 1987a, b).

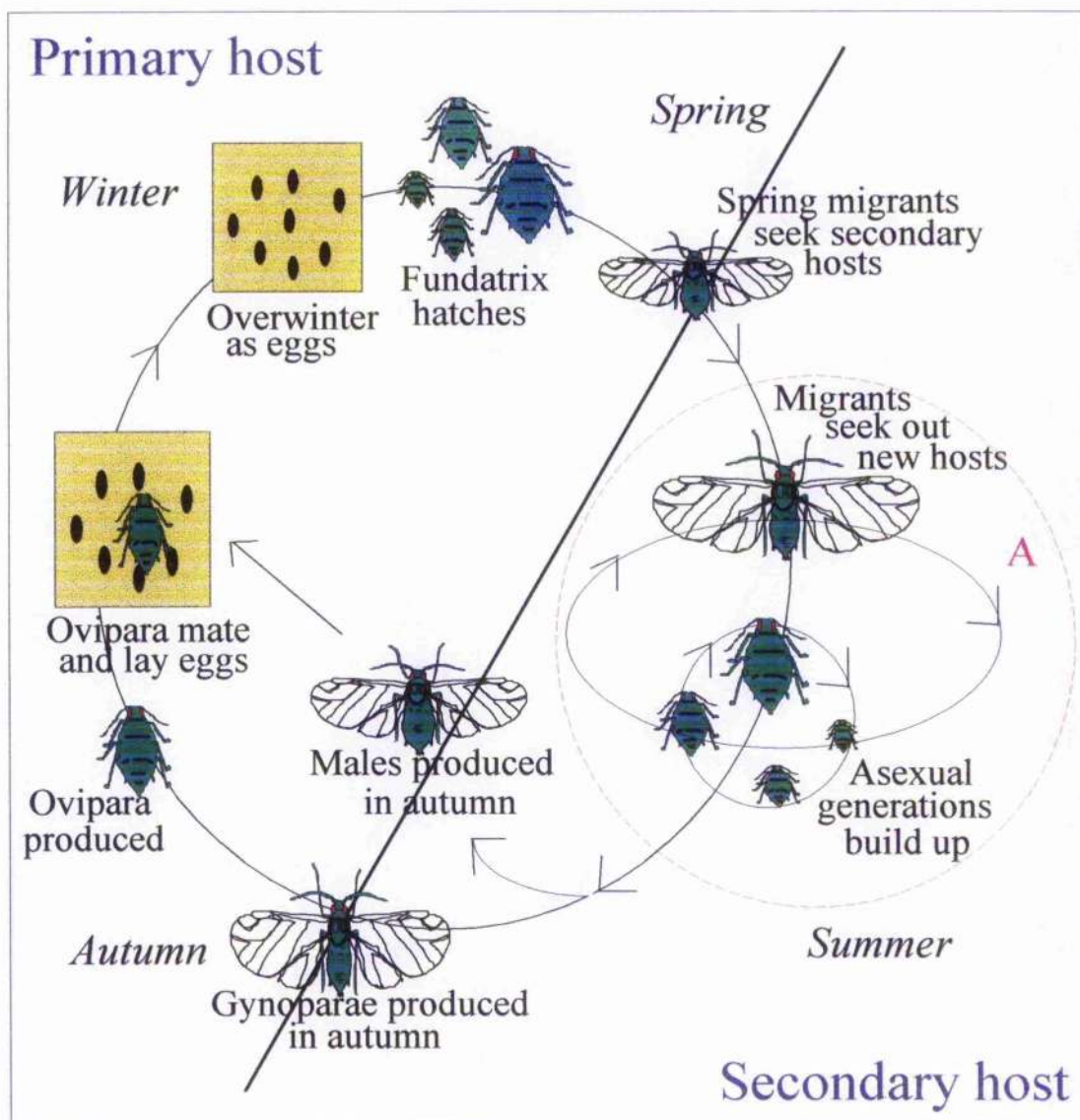
In Britain, aphids cause losses in field crops, horticulture and forestry through feeding damage, such as nutrient drain and gall formation, and the transmission of pathogenic plant viruses (Wellings *et al.*, 1989). The black bean aphid, *Aphis fabae* (Scopoli) is a highly polyphagous aphid which is considered important due to both its direct feeding damage of many bean species, *Vicia faba* sp., and its ability to vector viruses of sugar beet (Blackman and Eastop, 1984). In contrast, the peach potato aphid, *Myzus persicae* (Sulzer), does not cause extensive feeding damage under normal circumstances (Van Emden *et al.*, 1969), but it is also highly polyphagous aphid (Blackman and Eastop, 1984) that is able to transmit over 100 plant viruses throughout its host range (Brunt *et al.*, 1996). Among the viruses that *M. persicae* can transmit are many that are harmful to economically important crop species (e.g. Ragsdale *et al.*, 1994; Salazar, 1994), including potato virus Y (PVY) and potato leaf roll virus (PLRV) in potatoes, beet mild yellows virus (BMV) and beet yellow net (BYNV) in sugar-beet, and pea enation mosaic virus (PEMV) and pea (or bean) leaf roll (BLRV) in peas (Blackman and Eastop, 1984).

### 1.2.2 Aphid Lifecycle

The ability of aphids to reproduce rapidly has made the group highly effective as pests. All aphids can reproduce parthenogenetically, that is asexually, giving rise to live offspring. Due to the physiology of aphids, a parthenogenetic female can contain embryos which already contain their own developing embryos (Dixon, 1987). This is called 'telescoping' of the generations and is one of the main reasons why aphid numbers can increase rapidly. Except where mutation has occurred, aphids which are produced parthenogenetically through apomictic thelotoky are genetically identical to their mothers, as there has been no genetic recombination (Blackman, 1987; Hales *et al.*, 1997).

In many aphids parthenogenesis occurs within a reproductive cycle that alternates between sexual and asexual methods (Figure 1.1), and its occurrence is dependent on external cues, such as the time of year and host plant associations (Blackman and Eastop, 1984). However, some species of aphid, and some clones within species, are unable to reproduce sexually and only parthenogenetic reproduction remains throughout the year (Figure 1.1, section A) (Blackman, 1987). The reasons for loss of the sexual cycle are not well understood (Hales *et al.*, 1997). In these completely asexual aphids there is no opportunity for recombination to occur and, except for mutation events, genotypes are fixed (Hales *et al.*, 1997).

The production of alatae (winged forms) is important for the movement of



**Figure 1.1**

**General aphid life-cycle, showing host alteration and changes that occur in the method of reproduction with season. Panel A highlights the life-cycle of aphids which only have the ability to reproduce parthenogenetically. (Adapted from Blackman and Eastop, 1984)**

aphids over long distances. Movement from primary to secondary hosts is commonly achieved by flights of alatae, while the aphids which move back to the primary hosts in the autumn are males and gynoparae. The numbers and times of these alatae flights are often used to produce pest forecasts for agriculture (e.g. Halbert *et al.*, 1994; Wright *et al.*, 1995). The majority of parthenogenetic clones, even those without the ability to reproduce sexually, retain the ability to produce the different phenotypes of alatae and apterae (non-winged forms).

### **1.3 Current Methods of Invertebrate Control**

#### 1.3.1.1 Insecticides

Insecticides are the main aphid control measure currently in use. In the UK there are over 25 types of pesticide currently licensed for use against aphids, including organophosphates, carbamates, synthetic pyrethroids and fatty acids (Whitehead, 1998). Although insecticides are obviously popular, there are problems with their use. These include damage to other insect groups, as well as possible effects on human populations.

#### 1.3.1.2 Insecticide Resistance

Extensive and intensive use of insecticides has led to widespread resistance to these chemicals in many insects, including aphids (Devonshire, 1989 a, b). Resistance is deemed to have developed where exposure of a population to a dose of insecticide which used to give control, no longer proves to be adequate (Devonshire 1989 b).

In *M. persicae* a direct connection was found between insecticide resistance and an increase in the production of an enzyme, carboxylesterase E4 (Devonshire and

Sawicki, 1979). This enzyme both degraded and sequestered insecticidal esters (Devonshire and Moores, 1982) and conferred a broad range of resistance to organophosphates, carbamates and pyrethroids (Foster *et al.*, 1996). Resistant strains of *M. persicae* contained more copies of the E4 gene than insecticide susceptible populations (Field *et al.*, 1988). The strains also showed different levels of resistance depending on the number of copies of the E4 gene that they possessed (Sawicki *et al.*, 1980). A similar esterase-based resistance to insecticides has been detected in the hop aphid, *Phorodon humuli* (Lewis and Madge, 1984). In the cotton aphid, *Aphis gossypii*, resistance to organophosphates and/or carbamates has been conferred by mutated forms of acetylcholinesterase less sensitive to inhibition by the active insecticidal compounds (Devonshire and Moores, 1982). This form of insecticide resistance has also been found in *M. persicae* (Foster *et al.*, 1998). Several other species of aphid are now reported to display some level of insecticide resistance, although the mechanisms employed by many of the different species have not yet been investigated (Devonshire, 1989 b).

The increase in incidence of insecticide resistant aphid populations is closely correlated with circumstances where a large proportion of the population are under intensive insecticide selection, such as in glasshouses or where crops are subject to multiple spraying (Devonshire, 1989 a, b). In situations where control agents are mixed together, such as organophosphate/ pyrethroid mixtures, selection of resistant populations is more rapid than when an organophosphate is used by itself (French-Constant *et al.*, 1987).

Along with populations which have been selected for insecticide resistance by frequent chemical application, some 'natural' populations of aphids which had not previously been exposed to insecticides also showed high levels resistance (Devonshire

*et al.*, 1977). The conclusion drawn was that such resistant populations could only arise in the absence of pesticides if there were high rates of aphid immigration from areas under heavy insecticide selection. This rested on the assumption that the resistant populations of aphid would have some cost associated with the ability to perform in the presence of insecticides, which would in turn make them less "fit" than their susceptible counterparts in the absence of chemical control measures. Indeed, recent work carried out at the Scottish Crop Research Institute (SCRI) on local populations of *M. persicae* has shown that while the population structure may change during the growing season, possibly due to the application of pesticides, after winter the same genotypes predominate from year to year across crop types (Fenton *et al.*, 1998). The most prevalent genotype found at the beginning of the growing season over successive years was insecticide susceptible, therefore its numbers could be expected to fall after the application of pesticides. However, after each winter this genotype was restored to its former levels of incidence within the local population. This may be an indication of the adverse effects of pesticide resistance on the over-wintering ability of the aphids.

Insecticide resistance has indeed been implicated in a reduced ability to overwinter in the asexual form at very low temperatures ( $< 4^{\circ}\text{C}$ ) (Foster *et al.*, 1996; Foster *et al.*, 1997). It was suggested that this could be due to a reduction in the resistant aphids ability to respond to host plant cues which indicate that leaves are senescing, a signal which usually induces aphids to move onto other plant parts so that when the leaves fall off, the aphids are not separated from their food supply (Foster *et al.*, 1997). Insecticide resistant aphids also show a reduced ability to successfully transfer to sub-optimal host plants (Eggers-Schumacher, 1983).



However, insecticide resistant aphids are not always at a disadvantage when compared to their susceptible counterparts. When their reproductive levels and development times were compared at high (22-29 °C) or low (7-10 °C) temperatures, in the absence of insecticides, some resistant clones performed as well or better than susceptible clones on many host plants (Eggers-Schumacher, 1983; Lorriman and Llewellyn, 1983; Weber, 1985).

Some pests have been shown to have higher rates of reproduction after exposure to insecticides. Philippine populations of the brown planthopper, *Nilaparvata lugens*, had enhanced development rates after treatment with a variety of insecticides (Heinrichs *et al.*, 1982; Reissig *et al.*, 1982). This effect was not associated with a loss of planthopper predators. Furthermore, there is evidence that aphids can experience the same phenomenon, since aphids which survived treatment with organophosphates (Lowery and Sears, 1986a, b) or pyrethroids (French-Constant *et al.*, 1987) produced nymphs at a faster rate than untreated aphids of the same genotype.

The presence of insecticide resistant aphids on a crop means that a population of aphids remains unaffected or dies more slowly after exposure to insecticides. Where insecticides are used to prevent the spread of plant viruses, the presence of any residual aphids on crop plants can have serious consequences. For example, since 1992 it has been a statutory requirement to control aphids which transmit PLRV, PVY and tobacco vein necrosis (TVNV) viruses in crops grown under the Seed Potato Classification Scheme (Scottish Agricultural Science Agency, 1997). The presence of 0.26 % PVY or PLRV positive potato plants in a Super Elite crop can reduce the value from £200-350/tonne to as little as £40/tonne (Nickerson Seeds Ltd., pers. comm. 1998). These viruses can all be transmitted by *M. persicae*, an aphid in which insecticide resistance is now

relatively common (Foster *et al.*, 1998).

Concomitant with the general rise in insecticide resistance among many agricultural pests, there has been increasing public concern about pesticide use and the effects that these chemicals, and their breakdown products, may have on human health and the environment (Furst *et al.*, 1994; Semenza *et al.*, 1997). For example, the long-term effects of pesticides on human health have been shown to include an increased risk of cancer, particularly hematological cancer, neurotoxic effects, such as Parkinson's disease, and reproductive disorders (for a review see Baldi *et al.*, 1998). The environmentally detrimental aspects of several, previously popular, insecticides has led to their complete withdrawal from the approved insecticide list in Britain and many other countries: DDT is a classic example of this (e.g. Lacher and Goldstein, 1997; Mora, 1997). Both insecticide resistance and the potential for any currently used pesticide to be banned have led to more emphasis being placed on the development of alternative methods of pest control. This thesis is, in part, motivated by such considerations.

### 1.3.2 Tolerance To Insect Pests

Tolerance to insect damage occurs when a reasonable yield is obtained from a crop despite an insect infestation. While tolerance of damage is not strictly a method of insect control, it can be a viable and desirable alternative to the use of pesticides in some situations, such as organic farming. However, in general, tolerance is only acceptable in agricultural and other managed systems where no reduction in the value of the crop results (Kennedy and Barbour, 1992).

### 1.3.3 Avoidance of Insect Pests

Pest attack of plants can be reduced or eliminated by growing the plants in areas where, or at times when, their pests are either absent or present in very low numbers. For natural populations of plants the length of time necessary to achieve such avoidance methods is dependant on the reproductive cycle of the plant, on seed dispersal, etc. In managed systems, this strategy is commonly used. Agricultural systems also use crop rotation to avoid poorly mobile pests with long generation times, such as nematode pests (Kennedy and Barbour, 1992). For example, rotational gaps of five or more years are used to prevent undue yield losses through attack of *Globodera* sp. (Woll.) on potato crops (Phillips, 1989). One of the reasons for autumn planting of many crops, including barley and oilseed rape, was to avoid certain pests and pathogens at vulnerable stages of plant growth, however planting at this time of year exposed the crops to a different range of pests rather than avoiding them altogether. For example, winter and spring oilseed rape are both damaged by seed weevils and pod midge, but spring sown crops are subject to damage from stem weevil and winter sown crops are not (Graham, 1981; Moore, 1981). Conversely, winter sown oilseed rape is damaged by cabbage stem flea beetle, which is not a problem in spring sown crops (Graham, 1981).

### 1.3.4 Plant Resistance to Insect Pests

There are several plant features which can be employed in managed systems to

reduce invertebrate damage to crops. These attributes have been employed singly or in combinations, and their use forms the basis of many of the current strategies for controlling insect damage.

#### 1.3.4.1 Physical Characteristics

Plants may have physical characteristics which can deter pests. These include hardness or thickness of exposed tissues, habit of plant, surface waxes, shape and colour of leaves, presence of spines and hooks on leaf surfaces, etc. (Kennedy and Barbour, 1992). The number of trichomes (hairs or pubescence) present on plant surfaces has been directly correlated with resistance to insects in a number of plant species (Smith, C. M. *et al.*, 1994).

#### 1.3.4.2 Plant Surface Chemicals

Plants can emit chemicals which pest species use as cues for host plant selection, oviposition and feeding (Klingauf, 1987 a). Some of the volatile compounds produced by plants after insect attack have been shown to attract predators of pests (Price, 1981), however many insects attracted by plant volatiles are beneficial to the plants, such as pollinators (Panda and Khush, 1995). In addition, where repellents are produced, pests are often discouraged from alighting on the plant surface or from feeding, thus preventing or reducing damage (Gibson and Rice, 1989).

#### 1.3.4.3 Internal Plant Biochemistry

In the search for new ways to control pests, the exploitation of plant metabolites, which may have roles in plant defence mechanisms, has been considered. Plants

produce a variety of chemicals which are thought to have defensive roles, such as saponins, lectins, amylase inhibitors, proteinase inhibitors, and alkylating agents (Felton and Gatehouse, 1996). These compounds are postulated to act as antinutrients, toxins, or feeding repellents against attacking pests (Panda and Khush, 1995).

#### **1.4 Enhancement of Plant Resistance To Pests**

Resistance mechanisms to insects can be incorporated into crops in one of two ways; by standard breeding techniques or by transferring genes from sources of material that are unrelated to the crop. Traditional plant breeding is the most widely used technique.

Movement of a resistance trait into a crop by traditional plant breeding is achieved by crossing breeding lines of a plant with wild relatives or compatible species, and testing the resulting progeny for resistance to attack. Seed-banks and wild relatives of crops have proved to be a valuable resource for resistance (Auclair, 1989). However, problems can occur when different varieties or closely related species are used as the source of resistance traits for new crop cultivars. A plant breeding programme can disturb any equilibrium which exists naturally between the plant and the pests, by amplifying the plant alleles which confer resistance to the pests (Berenbaum and Zangerl, 1992). This will eventually lead to selection of compensatory alleles within the pest population, and the pests will adapt to the presence of the apparent resistance mechanism. Extensive or exclusive use of a particular plant resistance trait can also reduce its durability (Wilhoit, 1992) in the same way that overuse of pesticides has led to their reduced efficacy (see Section 1.3.1). For example, the wide use of raspberry

cultivars containing the A1 resistance gene, initially active against the large European raspberry aphid, *Amphorophora idaei* (Börner), led to the spread of a resistance-breaking gene throughout this aphid species (Birch *et al.*, 1994).

New biotechnological approaches offer alternatives to plant breeding as they allow the direct introduction into crop plants of resistance mechanisms derived from a diverse and unrelated range of genetic sources. Genetic engineering has successfully introduced genes encoding proteins from other plants, which have apparent pesticidal activity, such as plant protease inhibitors (PIs) and lectins (for a review see Estruch *et al.*, 1997). However, the level of pest control achieved with such transgenic plants has varied greatly (Ryan, 1990; Gatehouse *et al.*, 1993). For example, when snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) was expressed in transgenic potato plants, aphids under some test conditions survived for longer on plants expressing the lectin than on non-transformed and transformed non-GNA expressing controls (Down *et al.*, 1996).

Apart from incorporating pest resistance into crops, genetic engineering has also been used to generate plants which can produce functional animal proteins, including antibodies (Ma *et al.*, 1995). Expression of functional antibodies or active antibody fragments in plants may offer both a cheap method of antibody production and an easy way to protect vertebrates against diseases and pests (Whitelam and Cockburn, 1996). The genetic engineering and production of such proteins within plants also offers a way to produce intrinsic pest control factors that are not normally associated with controlling pests of plants (see Section 1.6).

When anti-invertebrate defensive mechanisms are moved into crops it is important to consider the natural range of defences already present in a plant,

particularly where transgenes are used. The successful insertion of a desirable gene into the plant genome does not guarantee that the gene product will be continuously available and there are several factors which can prevent or reduce both imported and endogenous gene function (Meyer, 1995). Epigenetic effects, and interactions with factors such as stress or plant age, can sometimes lead to gene inactivation by hypermethylation of the genes, or by inhibiting mRNA accumulation, thereby preventing gene transcription (Jorgensen, 1991). Such events have been observed for imported and endogenous genes in plants, where there is sequence homology between the two (Stam *et al.*, 1997). Where there is a high level of sequence homology between foreign and endogenous genes, there is an increased probability that any adverse reactions to the foreign gene will have similar effects on the endogenous genes; this is termed co-suppression (Jorgensen, 1991). For example, when a defence-related chitinase gene was inserted into the genome of tobacco plants with endogenous chitinases, some of the transformants had lower total expression of chitinases than non-transformed controls (Hart *et al.*, 1992). Thus, incorporation of foreign genes for resistance mechanisms, which have close endogenous homologues, into plant genomes may lead to reduced rather than enhanced pest resistance.

### **1.5 Pest Responses To Plant Resistance**

Careful consideration should be taken on the applicability of a chosen resistance mechanism to the target pest, as what is effective against one pest of a particular plant species may be ineffective against another. For example, when fed on transgenic tobacco plants expressing a potato PI, growth rates of *Chrysodeixis eriosoma*

(Lepidoptera) were reduced compared to controls (McManus *et al.*, 1994). Two other Lepidopterans, *Spodoptera litura* and *Thysanoplusia orichalcea*, fed on the same transgenic plants showed similar or enhanced growth rates, in comparison with controls. Also, a wide range of sensitivity towards a panel of lectins was found between aphid species regardless of their natural host plant range (Rahbé *et al.*, 1995), and some lectins were actually shown to enhance aphid growth and survival (Sauvion *et al.*, 1996).

There is now a large body of evidence for differences in reactions among aphid species and other insects to a range of plant derived compounds (Bur, 1985; Givovich and Niemeyer, 1995; Jongsma and Bolter, 1997; McManus *et al.*, 1994; Montgomery and Heinrich, 1974). With the current trend towards introducing complementary or foreign compounds into plants to increase their resistance to pests, such variation in response to ingested plant components among pest species is very important. However, while there are several theories for the basis of insect tolerance to plant compounds, or indeed the insecticidal actions of these compounds, most of the actual mechanisms have not yet been identified (Czapla, 1997; Felton and Gatehouse, 1996; Harper *et al.*, 1995; Rahbé *et al.*, 1995).

Levels of variation in response to plant resistance mechanisms, present within insect populations, should also be considered. A new cultivar of wheat initially screened with a laboratory strain of grain aphid, *Sitobion avenae* (Fabricius), showed favourable resistance to the aphid (Caillaud *et al.*, 1995). However, when exposed to a range of field-collected *S. avenae*, the wheat cultivar proved highly susceptible to aphid damage.

Many types of plant based resistance currently under investigation rely on the ingestion of plant material as either a discouragement from further attack, or as a means



of introducing toxic substances into the insect. The array of metabolites produced by a plant changes during its growth and development (Karban, 1992) and some metabolites can be induced after attack by insects (e.g. Felton *et al.*, 1989; Millar *et al.*, 1992). Alterations in the chemical constituents of the plant tissues may influence the feeding pests (Fritz, 1992) placing pressure on the insect gut to adapt. Many examples of adaptation have now been identified in insect guts, such as production of new PI-insensitive protease activity in response to changes in the dietary PI complement by some Lepidoptera and Coleoptera species (Jongsma *et al.*, 1995; Reeck *et al.*, 1997).

From studies of aphids and other pests, it is apparent that there are adaptive responses occurring in the gut after changes in their diets. In particular, polyphagous aphids encounter many distinct defence factors and, as has been found in other insects, aphids can respond to changes in the plant chemicals encountered with the production of more or different enzymes (Egaas *et al.*, 1991). Konarev (1996) has suggested that pests have co-evolved a broad range of digestive enzymes in response to defensive plant chemicals. However, for insects such as aphids, which use a food source that is very low in proteins, but high in free amino acids (Srivastava, 1987), maintaining digestive enzymes may not be an efficient expenditure of resources. Indeed, the number and variety of recorded aphid digestive proteases is very low (Srivastava, 1987). While the aphid gut is still the most likely site for a major adaptive response to dietary challenge, the possible mechanisms for adaptation have yet to be fully examined.

## 1.6 A Novel Target For Aphid Control

Pests of animals have been subject to the same types of control methods as their

plant pest counterparts. For example, organophosphate and pyrethroid insecticides are used for the control of livestock ectoparasites (French *et al.*, 1994; Kunz and Kemp, 1994). Many of the chemicals used as control agents are subject to resistance from the target pests (e.g. Hennessy, 1997; Kunz and Kemp, 1994; Waller, 1997), and there are concerns about environmental and consumer safety (e.g. Krzystyniak *et al.*, 1995; McEwen and McNab, 1997). However, an alternative strategy towards controlling pests of animals has been to specifically use pest antigens to confer resistance in the host (Billingsley, 1994).

Observations that cattle could naturally acquire resistance to tick infestation was first reported by Johnston and Bancroft (1918). Several workers have since found the presence of a host immune response to pest attack (e.g. Brossard, 1976; Smith *et al.*, 1993; Wikel, 1982). However, not every pest infestation leads to a protective response in the host.

In normal circumstances, animal hosts are thought to only come into contact with a limited number of pest antigens, mainly from the biting or piercing mouthparts, in saliva, or on the external surface of some internal pests. These antigens may not be sufficiently immunogenic to induce a response against all pest species or in all hosts, indeed they will almost certainly be under selection pressure to avoid detection by the host immune system.

Initially, crude extracts of pests were used to assess whether antigens which the host would not normally encounter, so called 'concealed' antigens, would produce protective immunity against specific pests. After feeding on rabbits immunised with internal organ extracts from the mosquito *Anopheles stephensi* (Alger and Cabrera, 1972) or the stable fly, *Stomoxys calcitrans* (Schlein and Lewis, 1976), the donor pests

showed pathological symptoms. Following these experiments, effective protection was achieved using gut preparations from several pest species, including the cat flea, *Ctenocephalides felis felis* (Heath *et al.*, 1994) and the human body louse, *Pediculus humanus humanus* (Ben-Yakir *et al.*, 1994). Vaccination with pest antigens gave a higher level of protection than was found after natural exposure to the same pests.

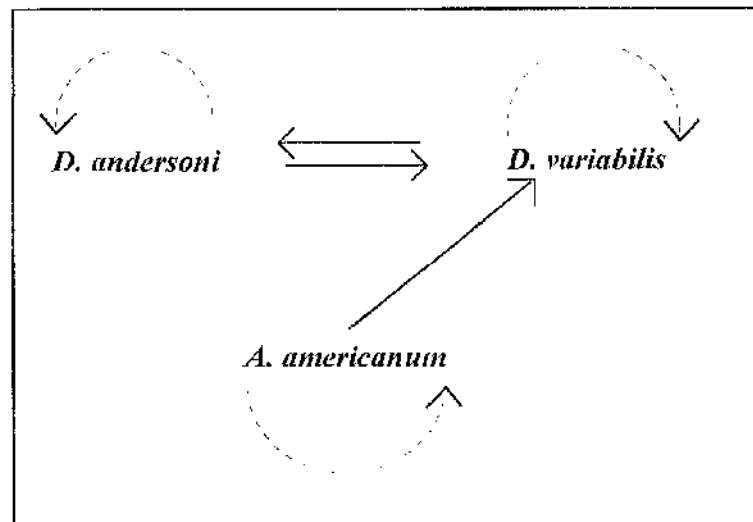
The use of gut antigens for immunisation does not necessarily mean that the gut was the primary target of the host immune system. However, there are now several examples of the pest gut as the active site of the protective response, including those against the cattle tick, *Boophilus microplus* (Cobon and Willadsen, 1990), the sheep blowfly, *Lucilia cuprina* (East *et al.*, 1993) and the sheep nematode *Haemonchus contortus* (Smith *et al.*, 1993; Smith, W.D. *et al.*, 1994). After careful fractionation and testing of the gut components, several protective antigens (PAs) have been identified in each of these species. The PAs characterised so far from *B. microplus* and *L. cuprina* have been glycosylated, which may be a protective feature against proteolytic degradation in their native gut environments. While the structure and location for many of the PAs from *B. microplus* (Riding *et al.*, 1994) and *L. cuprina* (Elvin *et al.*, 1996; Tellam *et al.*, 1992, Tellam *et al.*, 1994) characterised so far has been identified, their biochemical function has not. In contrast, one of the protective midgut antigens from *H. contortus* has been identified as an intestinal microvillar membrane aminopeptidase, termed H11 (Newton, 1995; Smith *et al.*, 1993).

The use of mosquito midguts as immunogens has not proved to be nearly as successful. Immunisation with crude gut extracts has produced varying results (Ingonga *et al.*, 1996; Ramasamy *et al.*, 1992), but some attempts have been made to try to characterise the potential protective antigens (Kwiatkowski and Marsh, 1997;

Ramasamy *et al.*, 1996; Ramasamy *et al.*, 1997). In the three examples described above (*B. microplus*, *L. cuprina* and *H. contortus*), the association between host and pest involves long-term and intimate contact (Wikel, 1982; Willadsen, 1997). In contrast, mosquitoes alight on their hosts for a brief time, and ingest a comparatively small blood meal (Wikel, 1982). The small size of the blood meal, limiting the amount of protective antiserum that the mosquito is exposed to, and the relative transience of mosquito contact with its host may be factors which reduce the potential of host immunisation with mosquito antigens to produce a protective effect.

Another possible reason for success in some cases, and failure in others, could be the environment within the pest gut. In both *B. microplus* and *H. contortus* there is constant and direct contact between the gut surface and the ingested food; in *L. cuprina* the food is always separated from the gut cell surface by the presence of a peritrophic membrane (PM) (East *et al.*, 1993). The structure of the PM in insects varies widely, but generally consists of a membranous layer or layers which line the insect gut, separating the ingested food from the surface of the gut cells (for reviews see Richards and Richards, 1977; Tellam, 1996; Terra, 1990). In all of these cases, the pests maintain a constant gut environment. In the mosquito however, the start of a blood meal triggers bursts of protease secretion and also the production of a PM (Billingsley and Rudin, 1992). Therefore, where both physical and biochemical gut conditions change rapidly, the location of, and interaction with, a suitable target antigen may prove to be very difficult to establish, and only low levels of protection would be expected. Conversely, where gut conditions remain relatively constant there may be a greater chance of achieving protective immunity.

An interesting observation across the many pests studied was the cross-reactivity, or absence of cross-reactivity, for antibodies between related pest species (Willadsen and Billingsley, 1996). For example, antisera raised against the midguts of the mosquito *Anopheles tessellatus* bound to a specific midgut protein and reduced their fecundity (Ramasamy *et al.*, 1992). The antisera against *A. tessellatus* showed considerable cross-reactivity with the gut of a related mosquito, *Culex quinquefasciatus*, but there was no accompanying reduction in fecundity. Similarly, guinea pigs with immunity to either of the ticks *Dermacentor andersoni* or *D. variabilis* were resistant to challenge by the other tick (McTier *et al.*, 1981). However, in the same study, resistance to another tick, *Amblyomma americanum*, was associated with cross-



**Figure 1.2**

**The immunity (---) and cross-immunity (—) of guinea pigs to three ticks (*D. variabilis*, *D. andersoni*, and *A. americanum*) following immunisation with antigens from each of the ticks (adapted from McTier *et al.*, 1981).**

resistance to *D. variabilis*, but not to *D. andersoni* (Figure 1.2).

To date, all the PAs identified in *B. microplus* and *L. cuprina* have been found to be glycosylated (East *et al.*, 1993; Willadsen *et al.*, 1995; Willadsen *et al.*, 1996). Deglycosylation of *B. microplus* PAs removed their protective activity (Lee *et al.*, 1991). The variation observed in the protective effect of antisera against related pest species indicates the absolute necessity for careful characterisation of the target gut antigens of the pest and assessment of the gut conditions. Scrutiny of gut proteins may reveal antigens, present in more than one pest species which fulfil the same function, and therefore offer a target for the control of several pests at once.

## **1.7 Analysis of Aphid Gut Proteins**

The importance of characterising target proteins has been stressed above, and while the structure of the aphid alimentary tract has been meticulously recorded (Ponsen, 1987; Terra, 1988, 1990) no detailed assessment has been made of the gut protein constituents in any aphid species. There are several ways to examine the proteins in any biological system and some of the options are discussed below.

### **1.7.1 Gel Electrophoresis**

Proteins migrate through different support matrices (acrylamide, cellulose acetate, starch, etc.) in electric fields at different rates, depending on their electrical charge and size. In non-denaturing buffers, separation by this method usually leaves enzyme function intact and enzymes can be visualised by specific staining using their

specific substrates. An alternative separation method uses the influence of a high voltage to move proteins through a pH gradient until they reach the point at which they have no net charge, i.e. the point within the pH gradient where the charges from positively and negatively charged amino acids cancel each other out. This is called the iso-electric point (IEP) of a protein and under a high voltage the protein will remain stationary within the gel matrix at its IEP. The separation of proteins by their IEP is called isoelectric focusing (IEF). After this method it is also possible to stain enzymes using their specific activity. The mobility and conformation of a protein can be modified by the presence of detergents and denaturants. In the presence of the detergent sodium dodecyl sulfate (SDS) the native charges of proteins are swamped and the proteins acquire an essentially negative charge. With the additional presence of a reducing agent which breaks disulphide bonds, the majority of proteins migrate through the gel matrix according to their size. One of the notable exceptions to this is glycoproteins, as SDS only binds to part of the molecule resulting in a lower net negative charge and artifactually high molecular masses due to anomalous migration during electrophoresis (Hames, 1990). However, as polyacrylamide gel concentration increases, for example in gradient gel electrophoresis, molecular sieving dominates over the charge effect and the glycoproteins approach their real molecular masses. The great benefit of these methods is that multiple samples can be separated on a single gel, allowing their direct comparison. One dimensional polyacrylamide gel electrophoresis (1DE) is the most commonly used method of protein analysis, but it does have limitations (Rickwood *et al.*, 1990). For example, what appears to be a band containing a single protein may actually be more than one protein of the same size migrating to the same point on the gel. In contrast, where a large indistinct band is assumed to be

formed from more than one protein, there may actually be only one protein which has different levels of glycosylation.

### 1.7.2 Enzyme Electrophoresis

Analysis of enzymes has been used to successfully differentiate within and between bacterial, plant and animal species in many studies (for a review see Menken & Ulenberg, 1987). However, when this method was employed to look at aphids, the majority of the studies found little or no variation within aphid species (Brookes & Loxdale, 1987; Simon *et al*, 1995). Only one previous study has tried to directly explore inter-specific variation in aphids using enzyme studies (Khuda-Bukhsh and Khuda-Bukhsh, 1991). Although restricted in scope, the authors concluded that there were differences between total enzyme profiles of the species under scrutiny.

Aside from the many technical restrictions in the examination of enzymes, if differences in the total aphid enzyme patterns are low, then variation in the sparse number of aphid gut enzymes could be expected to be even lower. Enzymes also represent a limited fraction of the total variation in any biological system. Furthermore, as with other selective methods, concentrating on the identification of enzymes imposes technical restrictions on the types of protein which can be examined, thereby overlooking other differences.



### 1.7.3 Two Dimensional Polyacrylamide Gel Electrophoresis (2DE)

The advent of two dimensional electrophoresis (2DE) in the mid-1970s allowed many of the problems of 1DE to be resolved. There are now a variety of sensitive methods for the separation of proteins using two different factors affecting protein movement in an electric field. The most successful variation has been separation by isoelectric point followed by separation using SDS PAGE (Anderson *et al.*, 1985; Fenton, *et al.*, 1985; O'Farrell, 1975).

To overcome some of the difficulties associated with being able to run only one sample per gel, and therefore assessing whether a protein is present in more than one sample, separate gels can be used in conjunction with a gel containing mixed samples (Fenton, 1993). If proteins are present in more than one sample, the pattern produced by sample mixtures after 2DE will reflect a lower level of complexity than would be obtained if each sample contained only unique proteins. For example, co-electrophoresis of two samples, each containing 10 common and 10 unique proteins, will produce a pattern of 30 proteins in total.

By combining 2DE with the sensitive technique of silver staining (Morrissey, 1981), the majority of proteins in a complex mixture can be visualised. The use of a polychromatic silver staining method, such as that of Morrissey (1981), has the additional benefit of colour differentiation between some proteins and the detection of minor proteins which might otherwise remain unobserved if another less sensitive staining method, for example Coomassie blue, was used.

## 1.8 Availability of Screening Material

One of the major problems in the screening and use of pest gut antigens is obtaining sufficient material to use as immunogen. Harvesting gut material from pests and then producing functioning fractions is extremely laborious, and requires culture of the pests *in vivo*, or *in vitro* where possible. Lack of material has been an enormous barrier to exploring the usefulness of many antigens, so a system which could produce large amounts of protective antigens would be highly desirable (Willadsen and Billingsley, 1996). The success of the vaccine against *B. microplus* was in part due to the production of antigen in economically viable quantities. This was achieved by expressing the target antigen as a recombinant protein in *Escherichia coli* and also in cultured insect cells (Willadsen *et al.*, 1995).

However, the exploitation of protective antigens from other organisms has been hindered by a continued reliance on material obtained from *in vivo* culture. For example, several potentially PAs have now been identified in the sheep parasite *H. contortus* (Longbottom *et al.*, 1997; Redmond *et al.*, 1997), but screening has been severely curtailed due to the lack of sufficient immunogen material to use for immunisation. Attempts have been made to produce functioning recombinant forms of the relevant proteins using *E. coli* and yeast expression systems, however the resulting proteins have lacked sufficient antigenicity to be useful (Redmond, pers. comm.). In many recombinant protein production systems, the biological activity of the translated protein may be lost as the protein does not adopt the correct three-dimensional structure and/ or is not properly glycosylated. Insertion of *H. contortus* genes into an *E. coli* expression system produced insoluble proteins without any glycosylation, while a very

limited amount of glycosylated protein was produced after expression of the same *H. contortus* genes in yeast (Redmond, pers. comm.).

An interesting new alternative to the previous two organisms is the use of potato virus X (PVX) as a vector to produce foreign glycosylated proteins in plants (Chapman *et al.*, 1995). The gene for the desired protein is fused to the gene of the PVX coat protein and the recombinant protein is produced either unfused within the plant tissue, or fused to the coat protein. Preliminary experiments, not described in this thesis, have shown that the PVX coat protein system may be useful as a means for producing correctly glycosylated *H. contortus* proteins, without the need for *in vivo* culture of these nematodes (Irving and Redmond, unpublished observations). If this method proves to be capable of producing large amounts of pest proteins with native three-dimensional structures, it will be possible to greatly speed up the antigen screening process in the search for protective pest antigens for immunisation and also remove the need for *in vivo* culture of many of the target pests.

## 1.9 Research Aims

Factors which appear to increase the success of an anti-parasite immune protection in animals (see section 1.6) have several parallels with aphids. Phloem sap is nutritionally poor, and to survive aphids must feed almost continuously (Douglas, 1993). This type of feeding directly exposes aphids to any deleterious factors in the phloem sap for prolonged periods. Furthermore, ingested food appears to be in direct contact with the cells on the surface of the aphid gut (Ponsen, 1987) and the internal

environment of the aphid alimentary tract appears to be very stable. The pH range of the aphid gut is between 6 and 8 (Ponsen, 1987; Srivastava, 1987), which is within the functional range of antibodies (Harlow and Lane, 1988). Since aphids have few, if any, digestive proteases with which to break down ingested proteins (Srivastava, 1987), extra proteins present in the phloem would have a good chance of remaining intact and acting on the aphid gut.

Plants cannot be immunised against their pests, but it is possible to genetically modify them to produce foreign proteins, such as antibodies or functional antibody fragments (Whitelam and Cockburn, 1996). Resistance to some plant viruses has been achieved by transforming plants to express viral proteins (Miller *et al.*, 1997) and cytoplasmic expression of an anti-viral antibody fragment gave protection against artichoke mottled crinkle virus in transformed tobacco plants (Tavladoraki *et al.*, 1993). Thus, from the research already carried out on the parasites of vertebrates and on transformation of plants to produce protection against pathogens, it would appear that antibodies expressed in plants against gut antigens could be developed as a method for the control of aphids.

To fully utilise the 'hidden antigen' approach as a method for obtaining aphid control, the aphid gut molecular components must be characterised to find antigens which are specific to the aphid species under examination, and whose disruption does not have adverse effects on non-target or beneficial organisms. The ideal target gut antigens would be those which are found in all aphid species all the time, or which are produced by all aphids as a result of specific host plant use. If, for example, a protein was identified as being produced only by the aphid species feeding on potatoes, it would

be possible to use the protein as a target. A control method aimed at such antigens would therefore provide the simultaneous control of multiple pest aphid species. Cross-reactivity of an antiserum raised against the antigens from one aphid species, with proteins from other aphid species, such as has been found for anti-tick antibodies (McTier et al., 1981), would help to identify any such universal aphid protein targets. Finally, as aphids can be maintained on artificial diets for several generations it is relatively easy to screen antibodies raised against aphid components for deleterious effects. All of these factors make aphids a useful model system for many plant pests.

### **1.10 Plan of Work**

1. Establish whether 2DE, followed by polychromatic silver staining, is a useful method for the examination of aphid proteins.
2. Examine the protein profiles of aphid tissues and establish whether there are any differences in the proteins found in different tissues.
3. Examine the proteins found in different species of aphid and assess the similarities or variations in proteins from different tissues between aphid species.
4. Assess the impact of dietary change on the proteins present in the aphid gut within an aphid species.
5. Prepare aphid antigens for production of anti-aphid antibodies.
6. Establish an artificial diet for long term culture of aphids.
7. Assess the effects of the anti-aphid antibodies on aphid performance using the artificial diet system.

## Chapter 2

### Materials and Methods

#### 2.1 Materials

##### 2.1.1 Chemicals

All general laboratory chemicals were of Analar quality and were purchased from Sigma Chemicals, Poole, UK or BDH, Poole, UK, unless otherwise stated. Radiochemicals were supplied from ICN Flow, Irvine, UK. All the water used was single-distilled mains water, unless otherwise stated.

##### 2.1.2 Plants

All plants were supplied from stock populations maintained at SCRI.

##### 2.1.3 Aphids

###### 2.1.3.1 Stock Aphid Cultures

Aphid stock populations were maintained on plants in sealed cages at 17 °C under a 16/8 h light/dark regimen. The following aphid species were maintained in long term cultures on the host plants indicated:- *Macrosiphum euphorbiae* (Thomas),

*Aulacorthum solani* (Kaltenbach), and *Myzus antirrhinii* (Macchiati) were raised on potato (*Solanum tuberosum*, var. Record); *Myzus persicae* were raised on potato and Chinese cabbage (*Brassica chinensis*); *Rhopalosiphum padi* were raised on barley (*Hordeum vulgare*); and *Amphorophora idaei* were raised on raspberry (*Rubus idaeus*).

#### 2.1.3.2 Field Collected Aphids

To prevent any contamination of the aphid stock populations with parasitoids or other aphid pathogens, all aphids collected from the field, such as *Brevicoryne brassica* (L.), were individually placed on excised leaves of the appropriate plant, in Blackman boxes. Where longer term culture of aphids was required, populations produced from healthy aphids were transferred onto individual plants in aphid proof cages. All Blackman boxes, etc., were maintained under the same lighting and temperature conditions as the stock cultures.

## **2.2 Methods**

### **2.2.1 Preparation Of Aphid Samples**

#### 2.2.1.1 Dissection Of Aphid Guts And Remnants

Apterae were dissected in 500  $\mu$ l of phosphate buffered saline (PBS) (9 mM  $\text{Na}_2\text{HPO}_4$ , 1.3 mM  $\text{NaH}_2\text{PO}_4$ , 0.14 M NaCl; pH 7.2) containing 1 mM EDTA and 2 mM Pefablok (a protease inhibitor: Boehringer Mannheim, Germany) (dissecting buffer) under a stereoscopic microscope (SDZ-PL: Kyowa, Tokyo) on a slide. Holding the aphid at the junction between the head and prothorax, a dissection needle was used



to disrupt the abdomen by removing the cauda and anal plate. The head was gently pulled away from the body, bringing the entire gut with it. Guts were kept on ice during dissection, and pooled in aliquots of 30. Remnants, i.e. those portions of the aphid remaining after the removal of all gut tissues and embryos (which contain gut tissue), were kept on ice, then pooled in groups of six. After isolation, aphid guts and remnants were snap frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until required. All samples were stored in 100  $\mu\text{l}$  of dissection buffer.

#### 2.1.1.2 Detergent Extracts Of Aphid Tissues

Samples of 300 guts isolated from Chinese cabbage fed *M. persicae* were added to a pre-weighed micro test tube and sufficient dissection buffer was added to make a 10 % w/v suspension. The samples were manually homogenised in the micro test tubes, using plastic micro tube pestles (Eppendorf, Hamburg, Germany). Following homogenisation the suspension was left to stand for 1 h at  $4^{\circ}\text{C}$ , after which it was spun at  $15,800 \times g$  in a microfuge for 10 min at  $4^{\circ}\text{C}$ . The supernatant (S1) was removed and the pellet was resuspended in PBS containing 0.1 % Tween 20 (homogenisation buffer). The suspension was left mixing at  $1.5 \times g$  on a Vari-Speed rotator (Taab Lab Equipment Ltd, Aldermaston, Berkshire) for 1 h at  $4^{\circ}\text{C}$ , then centrifuged a second time. After the supernatant (S2) had been collected, the pellet was resuspended in homogenisation buffer. The resulting solution was left mixing as before for 4 h at  $4^{\circ}\text{C}$ , then centrifuged as before and another supernatant (S3) harvested. This process was repeated to produce a fourth supernatant (S4). The washed pellet was finally resuspended (20 % w/v) in homogenisation buffer containing 2 % Triton X-100. This solution was thoroughly

mixed for 4 h at 4 °C and after a final spin the supernatant (S5) was collected. The protein contents of samples from S1-S5 and the final pellet (EP) were analysed using 1DE followed by silver staining (see Sections 2.2.7.1 and 2.2.7.3). After determining the protein concentration in the samples (see Section 2.2.2), the supernatants and final pellet were stored at -70 °C.

## 2.2.2 Determination Of Protein Concentration

Protein concentration was quantified using the method of Bradford (1976). Briefly, pre-made Bradford assay solution (BioRad, UK) was diluted 1 in 5 with distilled water. 240 µl of the diluted reagent were added to wells on a 96 well microtitre plate (Dynatech, Jersey) containing 100 µl of the samples to be assayed. The solutions were left for 5 min, then the absorbance was measured at 595 nm using a microplate reader (Dynatech MR7000: Dynatech, Jersey, CI). The absorbance of the samples was assessed by comparison to known concentrations of bovine serum albumin.

## 2.2.3 Polyclonal Antiserum Production

### 2.2.3.1 Preparation Of Whole Insect Immunogen

Whole *M. persicae* were collected from Chinese cabbage and snap frozen in liquid nitrogen in samples of 500 mg. After adding 200 µl of dissection buffer, each sample was put through three cycles of freezing in liquid nitrogen and manual grinding with a micro tube pestle (Eppendorf, Hamburg, Germany). To ensure that the samples

were completely disintegrated they were sonicated at 5 cycles per second, in a water-cooled cup-sonicator (Ultrasonic Disintegrator: Minisonix Inc, USA) for 10 min. The prepared whole aphid antigen was stored at -70 °C until required.

#### 2.2.3.2 Preparation Of Aphid Gut Immunogen

Samples of 300 guts from *M. persicae* raised on Chinese cabbage were ground, sonicated and stored following the same method as for the whole aphid antigen.

#### 2.2.3.3 Immunisation Protocol

Pre-immunisation blood samples from adult sheep, maintained by the Moredun Research Institute, Edinburgh, were taken. The sheep were then immunised with one injection of either whole aphid (500 mg of whole aphid per immunisation) suspended in 5 ml of Freund's complete adjuvant, or aphid gut immunogen (300 isolated guts per immunisation), suspended in 5 ml of Quil A adjuvant. This was followed by two further immunisations, three weeks apart, with the same immunogens and Quil A adjuvant, but changing Freund's complete adjuvant for Freund's incomplete adjuvant. After the final immunisation blood samples were taken from the sheep at fortnightly intervals. The antibody content of the samples was assessed on Western blots after 1DE (see Section 2.3.4).

#### 2.2.4 Artificial Diet (AD)

Several diets were tried for raising *M. persicae*, but many diets were only viable in the short term, lacking one or more vital components for the long term survival and

reproduction of the aphids. The diet described below was developed from the general principles described in work by Mittler and Koski (1976), but incorporating higher levels of tyrosine.

The diet was split in to four separate solutions. Two solutions (vitamin solution and trace element solution) were prepared in advance and stored at  $-20^{\circ}\text{C}$  for up to 3 months. Two solutions (amino acid & mineral mix solution and tyrosine in  $\text{H}_2\text{O}_{(\text{d})}$ ) were freshly made up for each batch of diet.

The final volumes of the four solutions in the diet were 1 ml of solution 1 ([100x] vitamin solution) + 100  $\mu\text{l}$  of solution 2 ([1000x] trace elements) + 50 ml of solution 3 ([2x] amino acid and mineral solution) + 40 ml of solution 4 (tyrosine solution), to give 100 ml total. The pH of the final solution was adjusted to pH 7.0 with KOH, prior to filter sterilisation by passing the diet through a sterile 0.45  $\mu\text{m}$  syringe filter (Nalge Co., Rochester, USA). Antibody or normal serum was added aseptically to the filter sterilised diet solution.

All ADs were presented to aphids in test chambers. Nescofilm (Bando Chemical Ind, Kobe, Japan) which had been sterilised for 5 min on a Chromato-Vue TM-20 ultraviolet transilluminator (UVP inc., CA, USA) was cut into strips 1.5 cm wide. All subsequent steps were carried out aseptically in a laminar flow cabinet at room temperature. Strips were stretched over the top of 24 mm lengths of clear acrylic tubing (external diameter of 30 mm and internal diameter of 25 mm: Plastics W Graham, Dundee, Scotland) which had been surface sterilised with 70 % alcohol. This formed the lower membrane through which the aphids could feed. The stretched membranes were sprayed with 70 % alcohol and left to dry. Using a dialysis pump, a small vacuum

was applied to the lower surface of the membrane forming a well into which 500  $\mu$ l of artificial diet was loaded. A second strip of Nescofilm was stretched across the top of the tube, excluding any air, and making a sealed diet sachet. Diet sachets were either used immediately or stored for up to 2 weeks at -20 °C.

#### 2.2.4.1 Aphid Diet Solutions

All solutions were made up in distilled water.

##### 1. Solution 1: [100x] vitamin solution

components	10 ml [100x]	final concentration
ascorbic acid	1 g	5.7 mM
biotin	1 mg	4.1 $\mu$ M
choline chloride	500 mg	360 $\mu$ M
folic acid	20 mg	45 $\mu$ M
meso-inositol	500 mg	2.8 mM
nicotinic acid	100 mg	810 $\mu$ M
pantothenic acid	50 mg	210 $\mu$ M
pyridoxine	25 mg	120 $\mu$ M
thiamine	25 mg	74 $\mu$ M
vitamin B12	10 mg	7.4 $\mu$ M

##### 2. Solution 2: [1000x] solution of trace elements

components	10ml [1000x]	final concentration
FeCl <sub>3</sub>	66.0 mg	41 $\mu$ M
CuCl <sub>2</sub> · 2H <sub>2</sub> O	14.5 mg	8.5 $\mu$ M
MnCl <sub>2</sub> · 4H <sub>2</sub> O	40.1 mg	20 $\mu$ M
ZnSO <sub>4</sub> · 7H <sub>2</sub> O	308.2 mg	107 $\mu$ M

## 3. Solution 3: [2x] amino acid and mineral solution

components	50 ml [2x]	final concentration
alanine	100 mg	11 mM
arginine	270 mg	15 mM
asparagine	550 mg	42 mM
aspartic acid	140 mg	10 mM
cysteine	40 mg	3 mM
glutamic acid	140 mg	9 mM
glutamine	150 mg	10 mM
glycine	80 mg	11 mM
histidine	80 mg	5 mM
isoleucine	80 mg	6 mM
leucine	80 mg	6 mM
lysine	120 mg	8 mM
methionine	80 mg	5 mM
phenylalanine	40 mg	2 mM
proline	80 mg	7 mM
serine	80 mg	8 mM
threonine	140 mg	12 mM
tryptophan	80 mg	4 mM
valine	80 mg	7 mM
citric acid	10 mg	0.5 mM
MgSO <sub>4</sub> ·7H <sub>2</sub> O	123 mg	5 mM
K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O	1.145 g	50 mM
sucrose	15 g	0.44 M

## 4. Solution 4: tyrosine solution

For 100 ml of artificial diet 60 mg of tyrosine was added to 40 ml H<sub>2</sub>O<sub>(d)</sub>. The solution was heated while stirring until the tyrosine dissolved, then allowed to cool before adding it to the other diet components. Tyrosine solution was made up fresh for each batch of diet. The concentration of tyrosine in complete AD solution was 3.3 mM.

### 2.2.5 Metabolic Labelling Of Aphid Gut Proteins

AD was made up following the standard protocol (described in Section 2.2.4), replacing methionine with radioactive labelled  $^{35}\text{S}$ -methionine. Twenty adult *M. persicae*, that had been feeding on artificial diet for two weeks, were placed onto diet sachets containing the labelled diet. These were allowed to feed for 14 h at room temperature, in constant light. At the end of the 14 h period the guts and remnants of these aphids were dissected and electrophoresed over-night on a 5-20 % 1DE. Half of the subsequent gel was silver stained and the other half was soaked for 30 min in Amplify (Amersham Life Sciences, UK) to convert the  $\beta$  radiation into signals detectable on film. The gel was then dried under a vacuum for 2 h on an LKB gel dryer with Whatman 1A filter paper as backing. The dried gel was placed next to a sheet of medical X-ray film (Fuji Photo Film CO, Tokyo, Japan) in an X-ray cassette with intensifying screen (Kodak, UK). The loaded cassette was stored for 2 months at  $-70^{\circ}\text{C}$  after which the X-ray film was developed. The silver stained portion of the gel was compared to the pattern of bands seen on the developed X-ray film, to assess which of the proteins were the result of *de novo* synthesis in the aphid gut over the course of feeding on the radio-labelled AD.

### 2.2.6 Isolation Of Immunoglobulin G (IgG) From Aphid Tissues

Freeze-dried Protein G sepharose beads (Sigma, UK) were swollen in 50 mM Tris (pH 8) containing 1 mM EDTA and 0.5 % Triton X-100 (TET buffer). Samples of

30 apterous *M. persicae* were fed for 6 days on AD containing 1:50 dilution of normal sheep serum or anti-*M. persicae* gut antiserum. The aphids were then dissected into head and abdomen portions on a dry microscope slide. After dissection the samples were manually ground as previously described (see Section 2.2.3.1) in 200 µl of TET buffer and then incubated with 200 µl of the swollen Protein G sepharose beads for 4 h, while mixing gently at room temperature. At the end of the incubation, 200 µl of 2x TET buffer was added and the samples were heated in boiling water for 4 min, in order to release the bound IgG. The samples were spun at 15,800 x g for 4 min and the supernatants were analysed for the presence of IgG on Western blots following IDE.

## 2.2.7 Analysis Of Aphid Proteins

### 2.2.7.1 One Dimensional Polyacrylamide Gel Electrophoresis (IDE)

Defrosted or fresh samples were put through three cycles of grinding and freezing in liquid nitrogen. After the final cycle, 100 µl of lysis buffer (3.12 mM Tris-HCl pH 6.75, 26 mM dithiothreitol, 2 % SDS, 10 % glycerol and 0.04 % bromophenol blue) was added to each sample, which were then boiled for 4 min. Samples were separated on either a 10 % or a 5-20 % acrylamide SDS-PAGE gel, using a discontinuous buffer system (Laemmli, 1970). A tris-glycine buffer (25 mM Tris, 0.2 M glycine: pH 8.7) was added to the tank and samples were electrophoresed for 16 h, using a Protean II XI cell (BioRad, UK). Each IDE included a 15 µl sample of molecular weight standards (Product no. M 6539: Sigma, UK) to allow assessment of protein molecular weight.



### 2.2.7.2 Two Dimensional Electrophoresis (2DE)

#### 1. Sample preparation

Samples were removed from storage and defrosted. Excess dissection buffer was removed by pulse spinning samples in a microfuge followed by careful removal of the buffer with a narrow bore pipette tip (Gel Saver pipette tips: Scientific Plastics, UK). Samples were resuspended in 100  $\mu$ l of lysis buffer (9.5 M urea, 2 % NP40, 5 % 2-mercaptoethanol, 2 % (v/v) ampholines (1.6 % 5-7, 0.4 % 3.5-10: Pharmacia, UK) and put through three cycles of grinding and freezing in liquid nitrogen. Prepared samples were either used immediately or stored at -70 °C.

#### 2. First Dimension - IEF gel electrophoresis

Isoelectric focusing (IEF) was done according to O'Farrell (1975), modified by Fenton (1987), using a Protean II tube cell and glass tubes with an internal diameter of 3 mm (BioRad, UK). The gel monomer solution consisted of 1 % (v/v) pH 3.5-10 ampholines, 9.2 M urea, 3.8 %/0.26 % (v/v) acrylamide/ bis acrylamide and 0.02 % (v/v) NP40 (BDH, UK). 0.001 % (v/v) ammonium persulfate and 0.01 % (v/v) TEMED were added to polymerise the acrylamide. Tube gels were pre-focused at 200, 300 and 400 V for 15, 30 and 30 min respectively using 0.01 M  $\text{H}_3\text{PO}_4$  as anode buffer and 0.02 M NaOH as cathode buffer.

When prefocusing was complete, samples (prepared as described in Section 2.2.1.1) were loaded on gels and run for 16 h at 400 V. Following IEF, gels were

removed from tubes by injecting distilled water around the edges of the gel to release it from the sides of the tube. The gels were placed in equilibration buffer, containing 10 % glycerol, 2.3 % SDS, 5 % 2-mercaptoethanol, 10 % 0.5 M Tris, pH 6.7, and 1 mM EDTA. After shaking for 30 min at room temperature, they were placed in fresh equilibration buffer and either used immediately for second dimension electrophoresis or stored at -70 °C.

### 3. Second Dimension - SDS PAGE

For each IEF gel a 5-20 % gradient SDS PAGE was used as the second dimension, according to the method of Laemmli (1970). Each IEF gel was sealed on top of the stacking gel with a minimum amount of 1 % agarose in equilibration buffer. The gels were electrophoresed for 16 h at 130 V. Each second dimension gel included a 15 µl sample of wide molecular weight standards (M 6539: Sigma, UK) placed in a small well in the agarose used to seal the IEF gel on top of the SDS PAGE, to allow assessment of protein molecular weight.

#### 2.2.7.3. Silver Staining

Proteins were visualised using the silver-staining method of Morrissey (1981). All steps were of 30 min duration and were carried out at room temperature in a fume hood, unless otherwise stated. After removal from the glass plates, gels were fixed by three consecutive washes in 50 % methanol/ 40 % water/ 10 % acetic acid, 5 % methanol/ 88 % water/ 7 % acetic acid, and finally 7.5 % glutaraldehyde. Gels were rehydrated with four washes in distilled water. After washing in a 5 µg/ml solution of dithiothreitol, the gels were incubated with 0.1 % (w/v) silver nitrate. Gels were rapidly

rinsed in a small volume of distilled water, followed by a rinse in a small volume of developer (3 % (w/v) sodium carbonate, containing 50 µl of 37 % formaldehyde per 100 ml). Gels were allowed to soak in fresh developer solution until development was stopped with the addition of an excess of 2.3 M citric acid. Gels were left gently agitating in the stopped developer for at least 1 h before being transferred to a solution of 0.03 % sodium carbonate (storage buffer) for longer term storage. Once stained, gels were kept in storage buffer and either stored as scanned images, using a ScanJet 4C/T (Hewlett Packard, Glasgow) or photographed.

#### 2.2.7.4 Western Blotting And Protein Detection With Antibody

Proteins which had been separated on 1DE or 2DE were Western blotted onto Hybond-C pure Nitro-cellulose (Amersham International, UK) using a Trans-blot cell (BioRad, UK) for 4 h at 252 mA, 74 V (Towbin *et al.*, 1979). After blotting, the nitro-cellulose membrane was blocked for 16 h, or over night, in 2 % bovine serum albumin (BSA) in PBS at 4 °C. The nitro-cellulose membrane was then incubated with anti-*M. persicae* antibody raised in sheep (1:500 v/v in PBS containing 2 % BSA) for 2 h at 18 °C. Following this incubation, the nitro-cellulose was washed for 4 x 5 min in PBS and then incubated with alkaline phosphatase conjugated anti-sheep IgG (1:4000 v/v; Sigma, UK) in PBS with 2 % BSA. The blot was washed for 4 x 5 min in PBS to remove unbound anti-sheep IgG. Antibody binding was detected by reaction of the alkaline phosphatase with 37.5 µl of 22 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 50 µl of 23 mM 4-nitroblue tetrazolium chloride (NBT) in 10 ml of 100 mM Tris, pH 9.5, containing 100 mM NaCl and 5 mM MgCl<sub>2</sub>. A 15 µl sample of pre-cocloured

standard molecular weights (MultiMark multi-coloured standards: Novex, USA) was included on each 1DE and 2DE gel to allow assessment of protein molecular weight after blotting.

#### 2.2.7.5 Western Blotting And Protein Detection With Biotinylated Lectins

Proteins which had been separated on either 1DE or 2DE were Western blotted onto Hybond-C pure Nitro-cellulose (Amersham International, UK) using a Trans-blot cell (BioRad, UK) for 4 h at 252 mA, 74 V (Towbin *et al.*, 1979). After blotting, the nitro-cellulose membrane was blocked for 16 h, or over night, at 4 °C in TNTT buffer (1 mM TRIS, 0.5 M NaCl, 250 µM Thimerosal and 0.05 % Tween 20) containing 1 % essentially globulin-free BSA. Following blocking, the nitro-cellulose was incubated with a biotinylated lectin (Vector Labs, UK) at 5 µg/ml in TNTT containing 0.5 % essentially globulin-free BSA, for 2 h at 18 °C. After this incubation, the nitro-cellulose membrane was washed for 4 x 5 min in TNTT, and then incubated with alkaline phosphatase conjugated streptavidin (1:4000 v/v: Sigma, UK). The blot was washed for 4 x 5 min in TNTT. Antibody binding was detected by reaction of the alkaline phosphatase with 37.5 µl of 22 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 50 µl of 23 mM 4-nitroblue tetrazolium chloride (NBT) in 10 ml of 100 mM Tris, pH 9.5, containing 100 mM NaCl and 5 mM MgCl<sub>2</sub>. A 15 µl sample of pre-coloured standard molecular weights (MultiMark multi-coloured standards: Novex, USA) was included on each 1DE and 2DE gel to allow assessment of protein molecular weight after blotting.

### 2.2.8 Analysis Of Protein Spots On 2DE

To assess the degree of protein similarity between different species, mixtures were used to detect co-electrophoresis of spots. Samples of guts, or remnants, of up to three different species were divided in two. Half of each sample was focused on its own and the other was mixed with the remainder of the other samples before being loaded on to a single tube gel. Following separation by the second dimension of 2DE (5-20 % gradient SDS-PAGE gel; Section 2.2.7.2) and silver staining (Section 2.2.7.3), the gel containing all three samples was used in conjunction with the others to identify those proteins which were:- 1. common to all species (appearing as 1 spot); 2. common to two (appearing as 2 spots); 3. unique (appearing as 3 spots); or 4. other permutations (absences, etc.). To minimise extraneous variation, the IEF and SDS-PAGE gels for comparisons of mixed samples were run simultaneously. To assess the molecular weights of the separated proteins, a 15  $\mu$ l sample of wide silver stain molecular weight standards (M 6539; Sigma, UK) was included on every second dimension gel.

To establish the reproducibility of the protein patterns, each sample type was analysed a minimum of six times and co-electrophoresed in at least three different mixtures. A map of all protein spots was constructed and each spot given a number. The map was used to make a qualitative assessment of variation and protein composition. To obtain dendrogram comparisons, spot numbers were arranged along the top of a table and each aphid species was arranged down the side. The presence of a particular spot in an aphid species was scored with a 1 and an absence with a 0. The matrix was then reformatted into a PHYLIP data file and analysed using the programmes DOLLOP and MIX (Felsenstein, 1995). Two types of parsimony analysis

were used to analyse the relationships produced by the protein data. Dollo parsimony assumes that a complex character state is more difficult to evolve than lose (Felsenstein, 1995), whereas MLX uses Wagner parsimony (Eck and Dayhoff, 1966; Kluge and Farris, 1969), which makes the assumption that a character is evolved at the same rate that it can be lost. Once the dendrogramatic relationships had been produced, bootstrap shuffling of the data was carried out, using protein absence/ presence as a discrete characteristic and parsimony dendrograms were produced linking those species which were most alike.

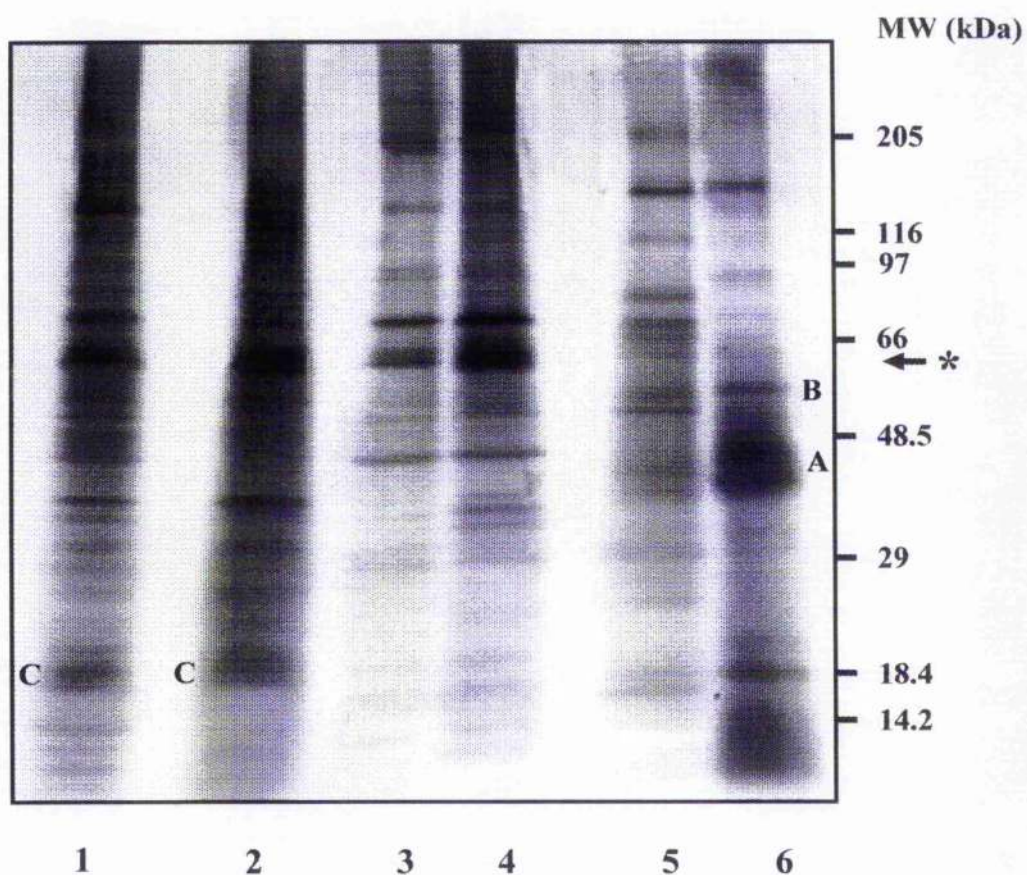
## **Chapter 3**

### **3 Tissue specific protein variation between aphid species**

#### **3.1 Introduction**

Only one previous study has explored inter-specific variation in aphids, using differences in total enzyme patterns in an attempt to examine species differentiation at the biochemical level (Khuda-Bukhsh and Khuda-Bukhsh, 1991). Although restricted in scope, assays using whole aphids showed differences between total enzyme profiles of the species under scrutiny. However, any analysis attempting to use total enzyme profiles to compare aphid species would be seriously limited since relatively few enzymes can be readily detected. Furthermore, enzymes represent only part of the total protein in any system and characterising enzymes necessarily ignores other components, such as structural proteins.

In this study protein electrophoresis and silver staining were combined to form a highly sensitive technique to examine similarities and differences in the protein composition of tissues from different aphid species. The protein variation across the aphid species examined was related to the use of gut proteins as potential aphid control targets, and also to the host-plant range of the aphids.



**Figure 3.1**

**Differences in protein profiles of two aphid species after separation using 1DE.**

Proteins were visualised using silver staining. The samples used were as follows: Track number (1) one whole *A. idaei*; (2) one whole *M. persicae*; (3) two *A. idaei* remnants; (4) two *M. persicae* remnants; (5) eight *A. idaei* guts; (6) eight *M. persicae* guts. Bands A and C have indistinct edges, indicating that they may be glycoproteins or that the bands are composed of more than one protein. \* marks the approximate molecular weight of symbionin. (Protein band B is one of the *M. persicae* gut proteins found to be actively produced by aphids after metabolic labelling; see Figure 3.2.2.)

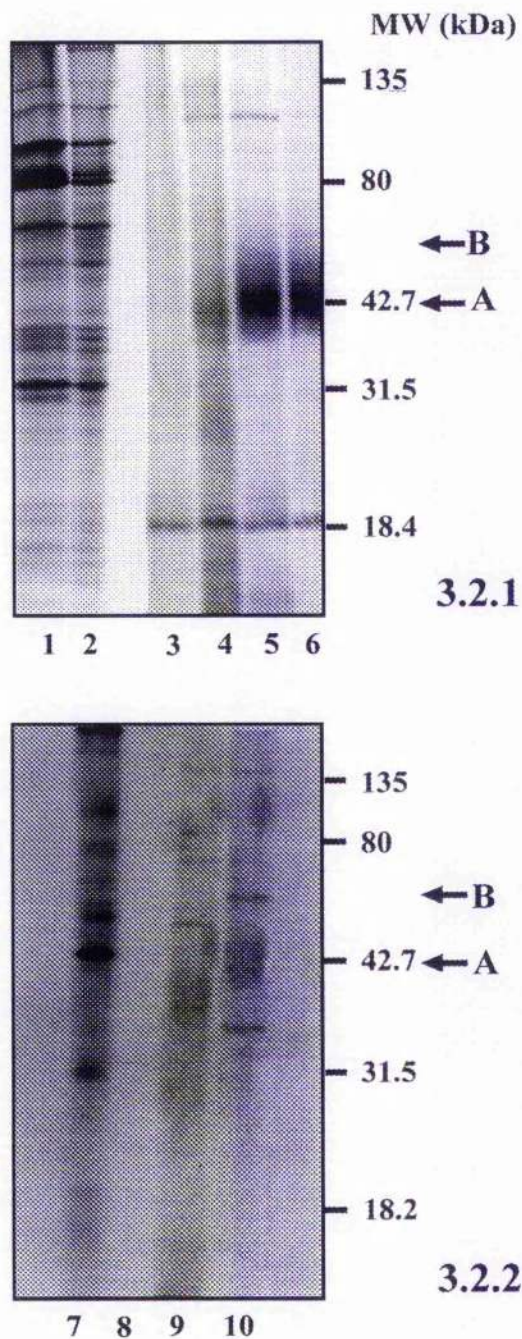


## 3.2 Results

### 3.2.1 One Dimensional Electrophoresis

Initial experiments separated the proteins from samples of aphid tissue using 1DE (Section 2.2.7.1) and the proteins were visualised with silver staining (Section 2.2.7.3). The results of 1DE separation and silver staining of proteins in whole, gut and remnant samples from *M. persicae* and *A. idaei* can be seen in Figure 3.1. The results showed that both remnant and guts possessed extremely complex protein patterns with approximately 85, 30 and 60 bands of protein observed in the whole, gut and remnant samples. Within each species there were distinct differences in the resolved proteins of gut and remnant samples (Figure 3.1: track 3 versus track 5 for *A. idaei*, and track 4 versus track 6 for *M. persicae* respectively). There were also clear differences in the proteins observed in the same tissues of the two aphid species (Figure 3.1: track 5 versus track 6), but very few discernible differences in remnant proteins (Figure 3.1: track 3 versus track 4). The complexity of the protein patterns in whole aphid samples meant that any differences between the species were not easily defined.

In some instances, the separation of proteins achieved after 1DE was not sufficiently marked to establish how many proteins of a particular molecular weight were present. For example, protein band C, indicated on Figure 3.1, is present in both whole aphid samples. However it was not clear whether there was one relatively abundant protein, several proteins of approximately the same molecular weight, or even whether the size of the band was due the presence of a glycoprotein (Hames, 1990).



**Figure 3.2**

**Metabolic labelling of aphid proteins from two aphid species.** After feeding on AD containing  $^{35}\text{S}$ -methionine *M. persicae* and *M. antirrhinii* were dissected. The proteins from remnant and gut samples were separated on 1DE and silver stained (Panel 3.2.1). Actively produced aphid proteins were labelled with  $^{35}\text{S}$ -methionine and were detected using 1DE followed by fluorography (Panel 3.2.2). The samples used were as follows: (1) two *M. antirrhinii* remnants; (2) two *M. persicae* remnants; (3) & (4) eight *M. antirrhinii* guts; (5) & (6) eight *M. persicae* guts; (7) metabolically labelled proteins from four *M. antirrhinii* remnants; (8) metabolically labelled proteins from four *M. persicae* remnants; (9) metabolically labelled proteins from sixteen *M. antirrhinii* guts; (10) metabolically labelled proteins from sixteen *M. persicae* guts. Proteins A and B are examples of proteins which are actively synthesised in *M. persicae* guts.

### 3.2.2 Metabolic Labelling Of Aphid Proteins

In insects, protein production in many tissues does not occur at a continuous rate throughout the life of the insect. This is due to the way in which insects grow, with life stages increasing in size through successive moults until the adult form is reached. Some insect tissues, such as the external cuticle, only show active protein synthesis during the moulting process (Happ, 1984). Aphids are hemimetabolous, undergoing incomplete metamorphosis from live birth, or egg, to adult form (Blackman, 1987). Indications of stage specific tissue production in aphids can be seen in the formation of wings only after the final moult. The aphid midgut also contains evidence of discontinuous protein production, as the lining of the oesophagus is shed into the midgut lumen at successive moults, where it can remain for the lifetime of the aphid (Moericke and Mittler, 1966). In contrast, there are obvious areas of continuous protein synthesis in aphids, such as the reproductive tract. Although many proteins were seen in all the tissue samples examined it was important to establish if these proteins were still being actively made by the aphids. Metabolic labelling, where a radioactive trace is attached to an essential amino acid to follow its active incorporation into proteins, was used to look at aphid protein production. Adult apterous *M. persicae* and *M. antirrhini* were fed for 14 h on AD containing radioactive labelled  $^{35}\text{S}$ -methionine (Section 2.2.5). These aphids were dissected (into remnants and guts) and the samples produced were processed as described in Section 2.2.5. Figure 3.2 shows the results of a metabolic labelling experiment. Incorporation of  $^{35}\text{S}$ -methionine into aphid proteins showed that active protein synthesis was occurring in both gut and remnant tissues. Aside from providing evidence of continued protein synthesis in the adult aphid, metabolic labelling



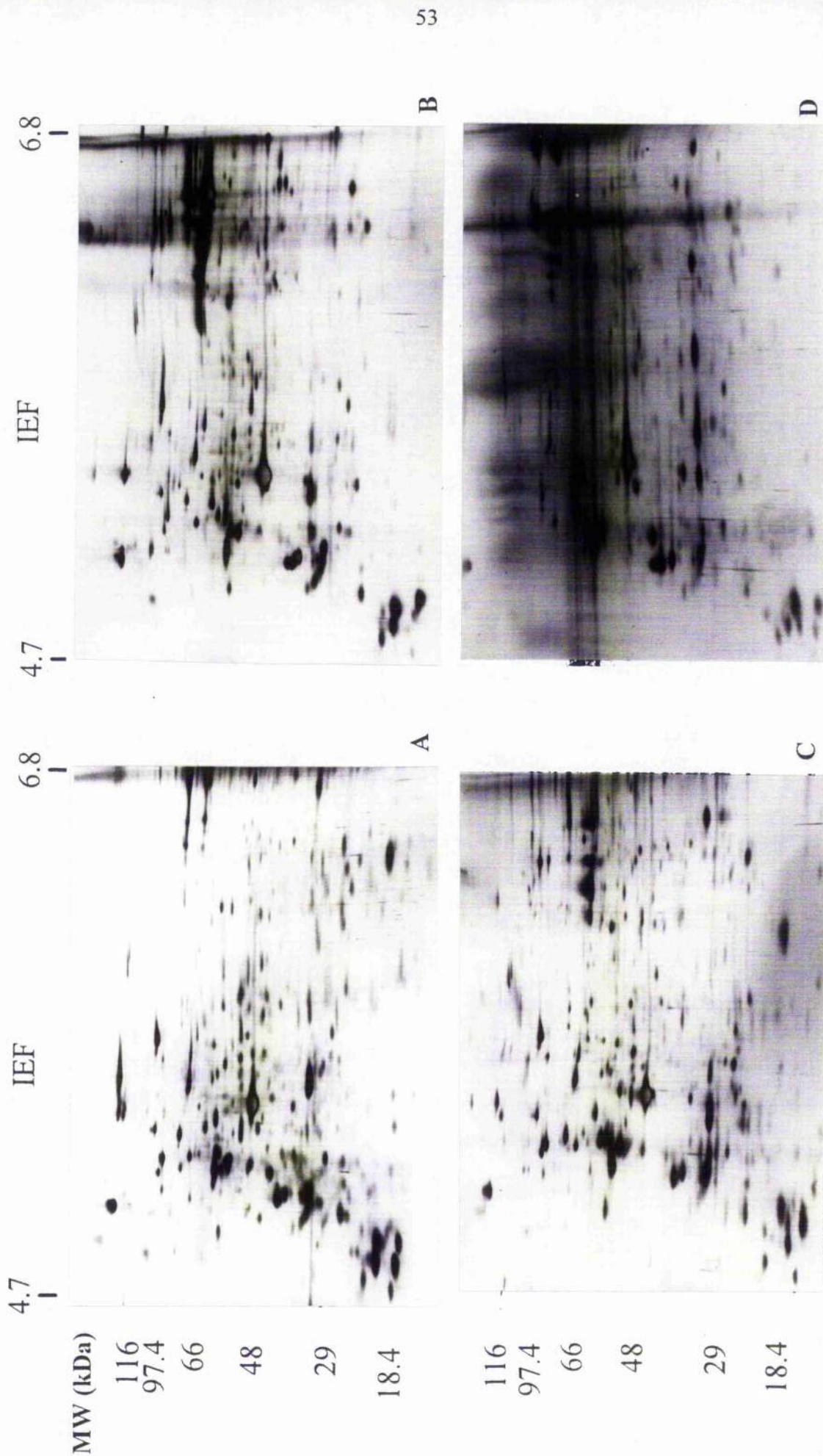


Figure 3.3

2DE of remnant proteins from three aphid species. Remnant samples consisting of six remnants were divided in two. Half of each sample was analysed on a separate 2DE gels. A. *R. padi*, B. *A. solani*, and C. *M. persicae*. The other half of each sample was analysed as a component of a mixture of all three sample (gel D). After silver staining, a combination of the results from individually electrophoreses samples and the mixture allowed the identification of those proteins common to two or more aphids, as well as those occurring in only one species.

also provided evidence that the proteins observed after IDE (Figure 3.1) were of aphid origin and not due to contamination of the samples with host plant proteins. For example, proteins marked as A and B on Figure 3.1 can be seen as metabolically labelled gut proteins in Figure 3.2.2, and they are therefore being actively synthesised in the aphids.

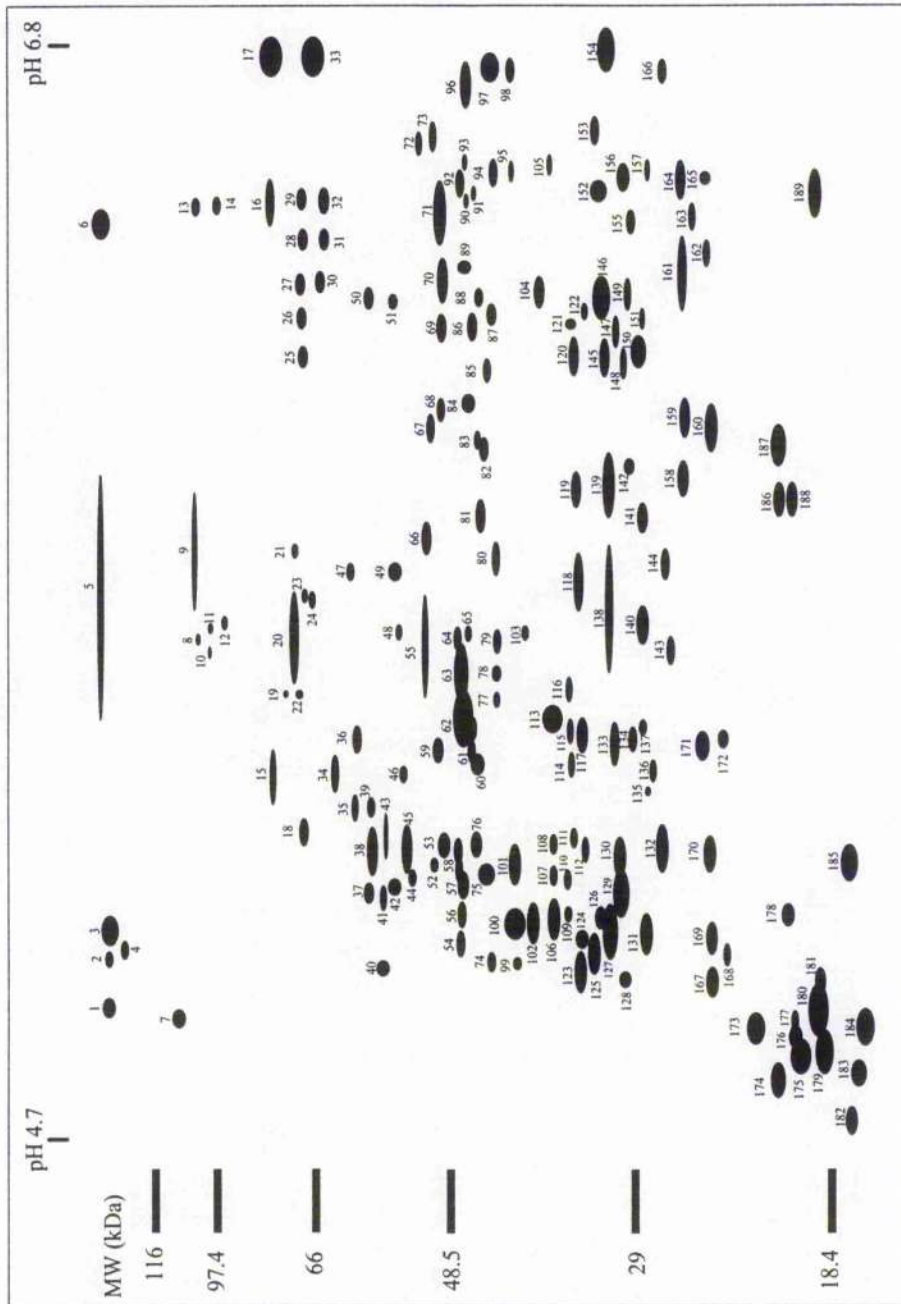
### 3.2.3 2DE

The 2DE method proved to be highly efficacious in the separation of the heterogeneous mixtures of proteins found in both the remnant and gut samples. Analysing samples individually and in mixtures established the exact position of many proteins, and also allowed the identification of proteins unique to each aphid species.

More than 180 remnant proteins, and over 50 major gut proteins, with isoelectric points (pI) of between 4.7 and 6.8, were resolved. However, there were proteins in both remnant and gut samples with pIs outside this range, some of which appeared as unfocused streaks at pI 6.8 (the origin in IEF) on the second dimension of 2DE (Figures 3.3 and 3.6). Proteins larger than 200 kDa and smaller than 6.5 kDa were not resolved using this experimental approach.

#### 3.2.3.1 Remnant Proteins

The remnant protein patterns from *Ma. euphorbiae*, (*M.e.*), *Au. solani*, (*A.s.*), *M. persicae*, (*M.p.*), *R. padi*, (*R.p.*), *A. idaei*, (*A.i.*), were examined, and over 150 proteins from each species were analysed using 2DE and silver staining (Section 2.2.7.2 and 2.2.7.3). Because of the very small numbers of *M. antirrhinii* (*M. a.*) available this



**Figure 3.4**

**Composite map of remnant proteins from five aphid species.** The remnant proteins from *M. persicae* (M.p.), *Ma. euphorbiae* (M.a.), *R. padi* (R.p.), *A. idaei* (A.i.), and *Au. solani* (A.s.) were mapped after separation by 2DE and visualisation with silver staining. The results were combined to produce a summary of all the major remnant proteins. The protein spots were given arbitrary numbers to facilitate identification of individual proteins within a protein profile.



#	Mp	Rp	As	Al	Me	#	Mp	Rp	As	Al	Me	#	Mp	Rp	As	Al	Me
1						49						97					
2						50						98					
3						51						99					
4						52						100					
5						53						101					
6						54						102					
7						55						103					
8						56						104					
9						57						105					
10						58						106					
11						59						107					
12						60						108					
13						61						109					
14						62						110					
15						63						111					
16						64						112					
17						65						113					
18						66						114					
19						67						115					
20						68						116					
21						69						117					
22						70						118					
23						71						119					
24						72						120					
25						73						121					
26						74						122					
27						75						123					
28						76						124					
29						77						125					
30						78						126					
31						79						127					
32						80						128					
33						81						129					
34						82						130					
35						83						131					
36						84						132					
37						85						133					
38						86						134					
39						87						135					
40						88						136					
41						89						137					
42						90						138					
43						91						139					
44						92						140					
45						93						141					
46						94						142					
47						95						143					
48						96						144					

Table 3.1

Remnant proteins present in the protein profiles of five aphid species. The remnant proteins from *M. persicae* (*M.p.*), *Ma. euphorbiae* (*M.e.*), *R. padi* (*R.p.*), *A. idaei* (*A.i.*), and *Au. solani* (*A.s.*), were mapped and the results combined to produce a summary of all the major remnant proteins (Figure 3.4). The protein spots were given arbitrary numbers to facilitate identification of individual proteins within a protein profile. Each species was then scored for the presence (■) or absence (□) of individual proteins. Those proteins which were unique to a species were indicated in grey (▒).

species was not included in the analysis of remnant proteins. Samples from each species were examined individually, and as components of mixtures of remnants from up to three separate species. An example of a 'three-way mix', plus the individual samples that made up the mix, can be seen in Figure 3.3 (Three sample mixture: Gel D. Individual samples: Gels A, B and C).

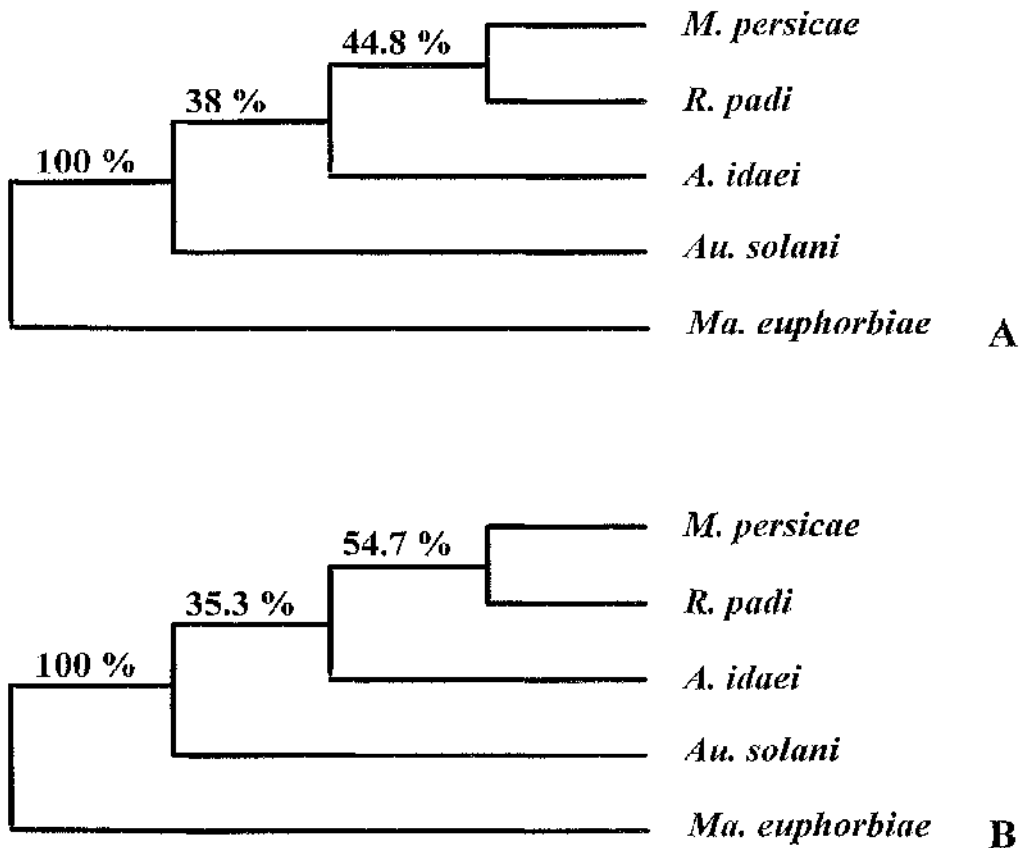
All the major, consistently observed, proteins from the remnants of each species were mapped. Using this information a compound map was made and each protein was assigned a number at random (Figure 3.4). When the remnants from each species were scored for the presence of the numbered proteins (Table 3.1: absence of a protein ☐ ; presence of a protein ☒ ), the extent of correspondence in protein position across the species could be seen. From a total of 189 major proteins 106 were common to all the

Aphid species	Remnant proteins	Gut proteins
<i>M. persicae</i>	2.6 %	54 %
<i>R. padi</i>	2.0 %	60 %
<i>M. antirrhinii</i>	/	53 %
<i>A. idaei</i>	0.7 %	65 %
<i>Ma. euphorbiae</i>	1.3 %	56 %
<i>A. solani</i>	0.6 %	59 %

**Table 3.2**

**Proteins found to be unique to individual aphid species.** After scoring both remnant and gut samples for their major protein content, the percentage of proteins found in only one species was calculated. The level of species unique proteins is far lower in the remnants than in the guts, for all species examined.





**Figure 3.5**

**Dendrograms produced following Dollo and Wagner parsimony of remnant protein data.** The data for this analysis is summarised in Table 3.1. After the analysis had been carried out, the data were shuffled using bootstrap analysis (100 steps), where protein presence/absence was treated as discrete morphology, to give an indication of how often the relationships described were formed during shuffling (%). No clear groups were formed and the relationships constructed between the species were not strong. Dendrogram A: Dollo parsimony analysis of remnant data, where *Ma. euphorbiae* was the outgroup. Dendrogram B: Wagner parsimony analysis of remnant data, where *Ma. euphorbiae* was the outgroup.

aphid species and a further 30 proteins were present in four out of five species. This means that 56 % of all the proteins were present in all five species and 72 % of the observed proteins were found in four out of five species. Along with the high levels of remnant protein conservation, a correspondingly low number of proteins were found to be unique to any of the five species examined (Table 3.2). Aside from two proteins found only in aphids fed on potato (Figure 3.4 and Table 3.1: proteins 89 and 107), there appeared to be no pattern for the absence or presence of proteins, so further analysis was carried out by constructing dendrograms from the data.

After the protein patterns of remnants had been summarised, dendrograms were prepared using MIX and DOLLOP analyses (Section 2.2.8). The resulting dendrograms are shown in Figure 3.5, with bootstrap percentages on each division within the dendrograms showing how often the relationships were formed during data shuffling. Although the two programmes used to create the dendrograms made different assumptions about the data and underlying conditions, they both gave the same relationships between species. In both cases, the aphids were not separated into clear groups. While there is some suggestion of a grouping of *M. persicae* and *R. padi*, neither this, nor the other relationships are highly significant (as indicated by bootstrap values).

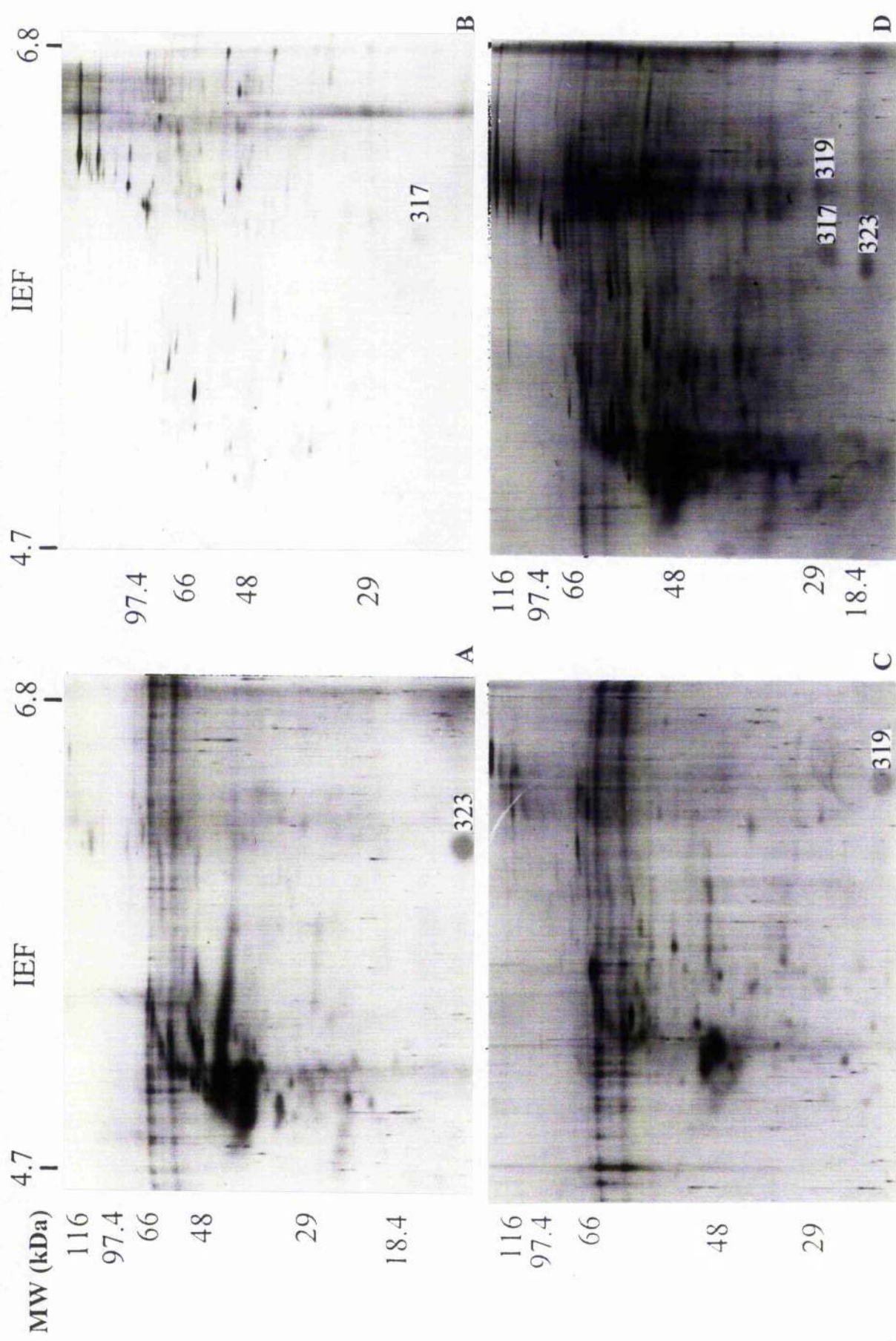


Figure 3.6

Gut proteins from three aphid species separated using 2DE. Gut samples consisting of 30 guts were divided in two. Half of each sample was analysed on a separate 2DE. Samples are A. *Au. solani*, B. *A. idaei*, and C. *R. padi*. The other half of each sample was analysed as a component of a mixture of all three samples (gel D). Following 2DE, gels were silver stained. The proteins 317, 319 and 323 are numbered according to the scheme in the compound diagram of gut proteins (Figure 3.7) and all are stained yellow after polychromatic silver staining with a characteristic rounded shape. All three of these proteins can be identified in the mixture of all three samples on gel D, and individually in gel A (323), gel B (317) and gel C (319), illustrating possible homologues of one protein.

### 3.2.3.2 Gut Proteins

Using the same procedure as for the remnants, the aphid gut proteins of six species were compared. Figure 3.6 shows of a mixture of gut samples from three aphid species after 2DE and silver staining (Figure 3.6, Panel D), and gut samples from the three individual species (Figure 3.6, Panels A, B and C). There were many more proteins present in the gut sample mixture than in any individual sample, indicating that there were more differences between the guts of these species than was found in their remnants. In fact, while each species had between 0.6 % and 2.6 % unique remnant proteins, this rose to between 53 % and 65 % for gut proteins (Table 3.2). A compound map of the major gut proteins from the six species of aphid was constructed from the combined results of over 48 2DE gels, as well as 12 mixtures (Figure 3.7), in the same way as the remnant protein map (Figure 3.4). This revealed that, unlike the remnants where no individual or group of proteins predominated, there were large amounts of 4 or 5 proteins. One such group of major gut proteins is present at approximately the same place on 2DE in all the aphids examined (Figure 3.7: protein spots 331-348), all of which had unfocused tails usually associated with glycosylation of the protein (Anderson *et al.*, 1985).

Variation of another major gut protein, from 14 to 18.5 kDa and pI from 5.3 to 6.4, was characterised in more detail on Western blots. Proteins from four aphids (*M. persicae*, *R. padi*, *M. antirrhinii* and *Mu. euphorbiae*), were separated on 1DE, blotted and probed with an anti-*M. persicae* gut antiserum (Figure 3.8). For each species, a different single protein band of between 14 and 18.5 kDa was recognised by the



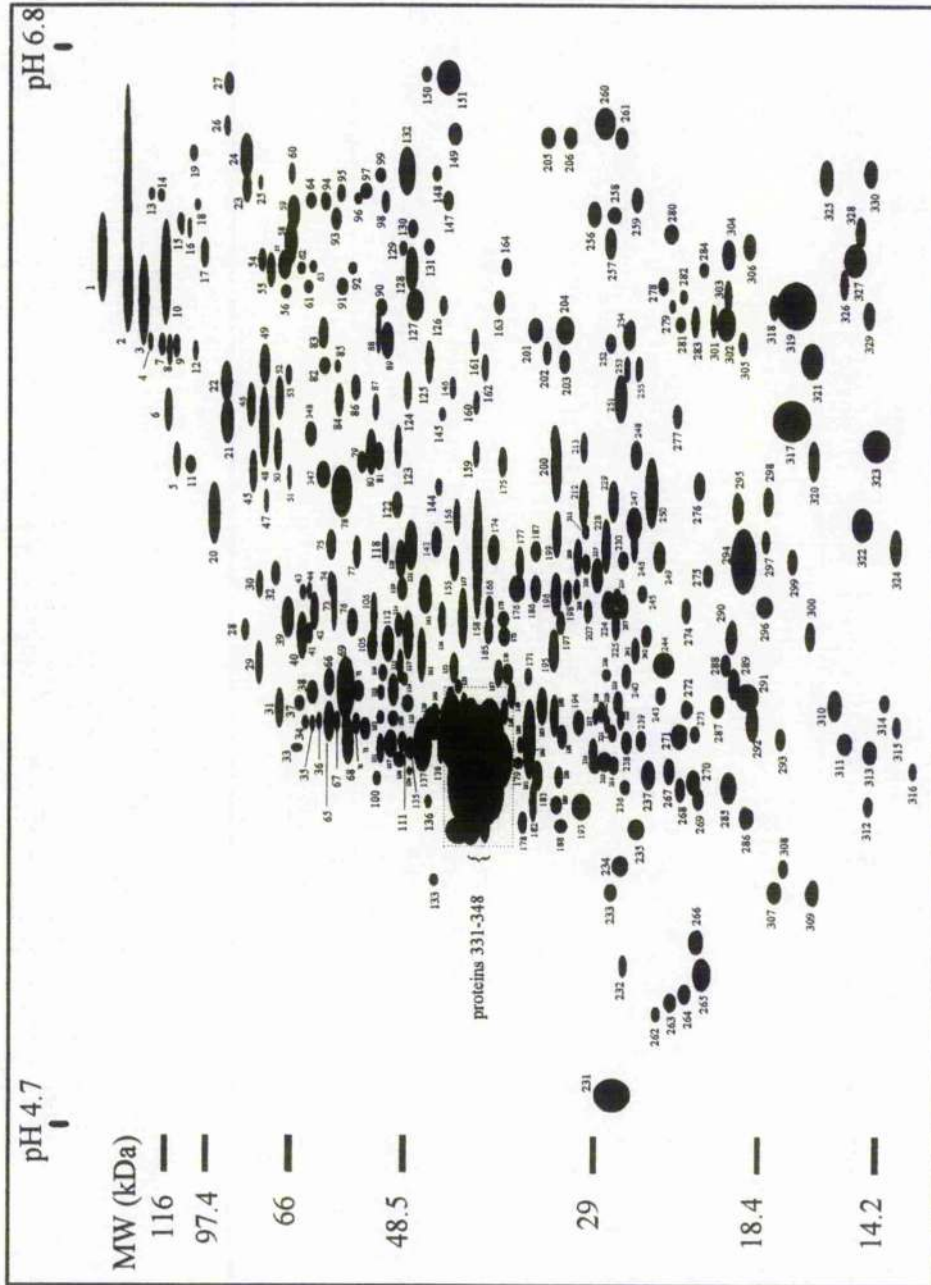


Figure 3.7

Composite map of gut proteins from six aphid species. The gut proteins from *M. persicae*, *M. antirrhini*, *Ma. euphorbiae*, *R. padi*, *A. idaei*, and *Au. solani* were mapped after 2DE separation and visualisation with silver staining. The results were combined to produce a summary of all the major gut proteins. The protein spots were given arbitrary numbers to facilitate identification of absence or presence of individual proteins in each species.



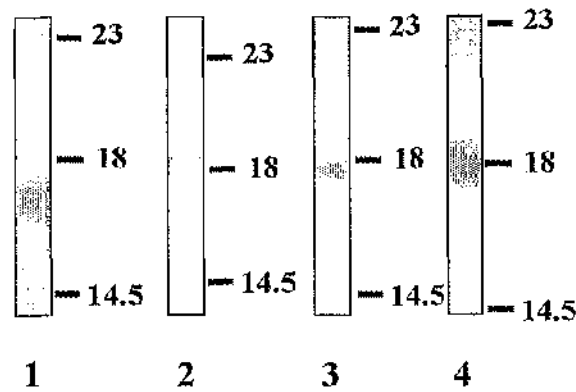
#	Ma	Ro	As	Al	Me	Ma	#	Ma	Ro	As	Al	Me	Ma	#	Ma	Ro	As	Al	Me	Ma	#	Ma	Ro	As	Al	Me	Ma	#	Ma	Ro	As	Al	Me	Ma	#	Ma	Ro	As	Al	Me	Ma	#
1							89							133								177						221								265					309	
2							90							134								178						222							266					310		
3							91							135								179						223							267					311		
4							92							136								180						224							268					312		
5							93							137								181						225							269					313		
6							94							138								182						226							270					314		
7							95							139								183						227							271					315		
8							96							140								184						228							272					316		
9							97							141								185						229							273					317		
10							98							142								186						230							274					318		
11							99							143								187						231							275					319		
12							100							144								188						232							276					320		
13							101							145								189						233							277					321		
14							102							146								190						234							278					322		
15							103							147								191						235							279					323		
16							104							148								192						236							280					324		
17							105							149								193						237							281					325		
18							106							150								194						238							282					326		
19							107							151								195						239							283					327		
20							108							152								196						240							284					328		
21							109							153								197						241							285					329		
22							110							154								198						242							286					330		
23							111							155								199						243							287					331		
24							112							156								200						244							288					332		
25							113							157								201						245							289					333		
26							114							158								202						246							290					334		
27							115							159								203						247							291					335		
28							116							160								204						248							292					336		
29							117							161								205						249							293					337		
30							118							162								206						250							294					338		
31							119							163								207						251							295					339		
32							120							164								208						252							296					340		
33							121							165								209						253							297					341		
34							122							166								210						254							298					342		
35							123							167								211						255							299					343		
36							124							168								212						256							300					344		
37							125							169								213						257							301					345		
38							126							170								214						258							302					346		
39							127							171								215						259							303					347		
40							128							172								216						260							304					348		
41							129							173								217						261							305					349		
42							130							174								218						262							306					350		
43							131							175								219						263							307					351		
44							132							176								220						264							308					352		

Table 3.3

Gut proteins present in the protein profiles of six aphid species. The gut proteins from *M. persicae* (M.p.), *M. antirrhini* (M.a.), *Ma. euphorbiae* (M.e.), *R. padi* (R.p.), *A. idaei* (A.i.), and *Au. solani* (A.s.) were mapped and the results combined to produce a summary of all the major gut proteins (Figure 3.7). The protein spots were given arbitrary numbers to facilitate identification of individual proteins within a protein profile. Each species was then scored for the presence (■) or absence (□) of individual proteins.

anti- *M. persicae* gut antiserum. The aphids used as antigen source for the antibody were raised on Chinese cabbage, so the cross-reactivity seen is independent of both species and/or host plant. These proteins can also be identified in individual species after 2DE separation and silver staining (for example -Figure 3.6: proteins 317, 319 and 323). After 2DE and polychromatic silver staining, these protein spots have approximately the same size, shape and colour (large, round and stained pale yellow). The proteins found in all six species can be seen in the summary figure (Figure 3.7) and are identified in the summary table of aphid gut proteins (Table 3.3) as proteins 323 (*Au. solani*), 294 (*M. persicae*), 321 (*Ma. euphorbiae*), 319 (*R. padi*), 322 (*M. antirrhinii*), and 317 (*A. idaei*). Based on the evidence from Western blots and 1DE, the proteins found in the different species could be alternative, homologous forms of the same molecule. However, further information about the cross-reactivity of these proteins with the anti-gut antiserum following 2DE would be necessary to confirm the presence of such homologous proteins.

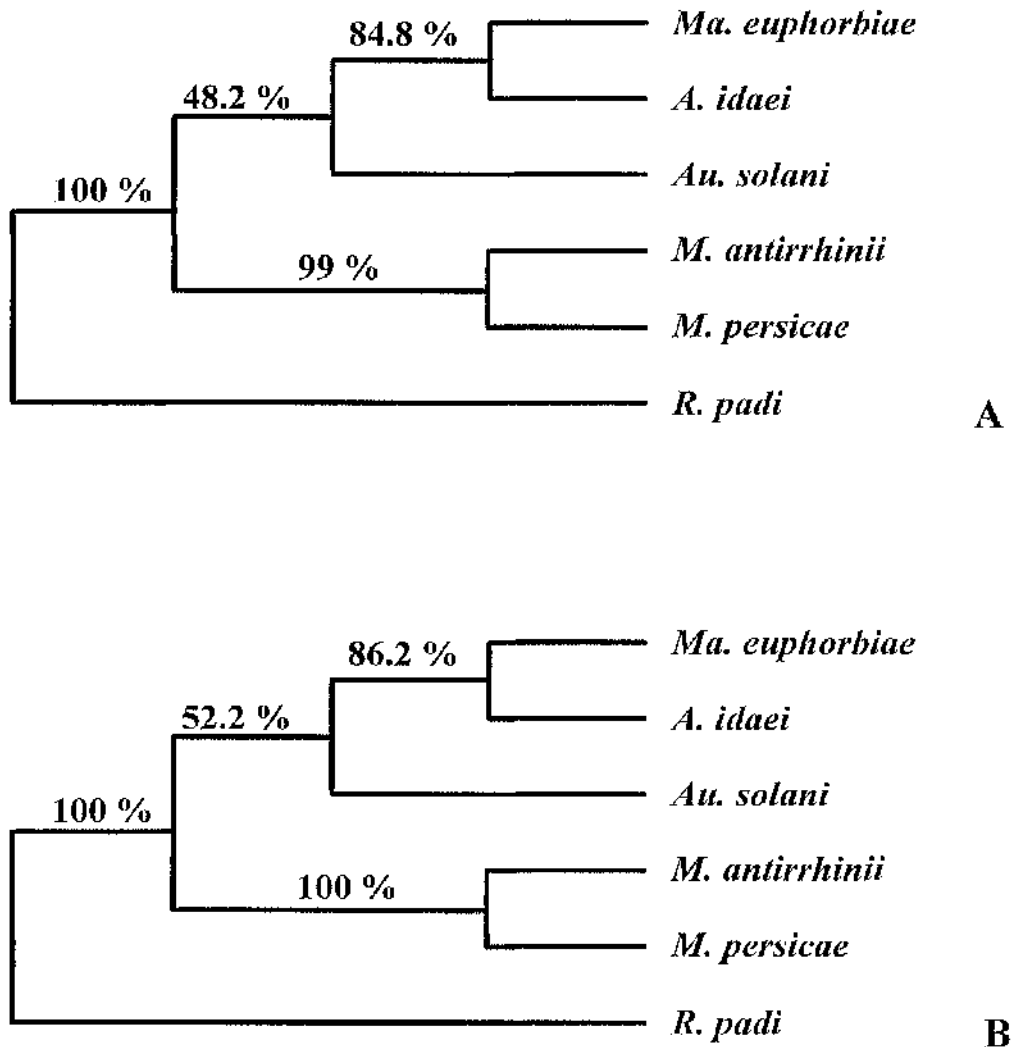
The high levels of inter-specific variation in gut tissue were analysed by scoring the presence and absence of all the numbered protein spots (Table 3.3). Using MIX and DOLLOP analysis of the gut protein data, dendrograms were formed (Figure 3.9). After bootstrap shuffling, the branches formed were more significant for the gut data than were found for the remnant protein data (as indicated by bootstrap values). Again, both types of parsimony used to analyse the gut protein data gave the same relationships and approximately the same bootstrap values. The aphids fell into two main groups, with *Ma. euphorbiae* and *A. idaei* in one group and *M. persicae* and *M. antirrhinii* in the second. *Au. solani* was linked with the group containing *Ma. euphorbiae* and *A. idaei*, while *R. padi* appeared to be distinct and separated from the other five aphid species.



**Figure 3.8**

**Identification of low molecular weight proteins unique to different aphid species.** Proteins from whole aphid samples were separated on 1DE, Western blotted and probed with anti-*M. persicae* antibodies. The aphid samples used were as follows:- Lane 1, two whole *M. antirrhini*; 2, two whole *M. persicae*; 3, two whole *Ma. euphorbiae*; 4, two whole *R. padi*. A single band of between 14.5 and 18.5 kDa was identified in each of the species examined. These proteins appear to corresponded to proteins identified as being species specific after 2DE separation and silver staining of gut proteins from 6 aphid species (proteins 322, 294, 321 and 319 respectively in Figures 3.6 and 3.7.), but confirmation of the identity of the proteins would have to be obtained on two dimensional Western blots.





**Figure 3.9**

**Dendrograms produced following Dollo and Wagner parsimony of gut protein data.** The data for this analysis is summarised in Table 3.3. To give an indication of how robust the relationships were, the data was shuffled using bootstrap analysis (100 steps). Protein presence/absence was treated as discrete morphology and the number of times that any groups were formed was given as a percentage. Two distinct groups were formed in both dendrograms and the relationships were significant (as indicated by %). Dendrogram A: Dollo parsimony analysis of gut data, where *R. padi* was the out group. Dendrogram B: Wagner parsimony analysis of gut data, where *R. padi* was the out group.

It should be noted that a comparison of the gut protein profiles of the four aphid species fed on the same cultivar of potato found no proteins common to all species. This would indicate that host plant protein contamination of samples was not a factor in the variation of aphid gut proteins, and this is consistent with observations of the low protein content of phloem (Srivastava, 1987; Douglas, 1993).

### 3.2.4 Protein Identification

While the identification of individual proteins in either the remnants or the guts was beyond the scope of this study, it was possible to eliminate some candidates. The predominant endosymbiont protein, symbionin, was identified in *M. persicae* mycetocytes and haemolymph, as a protein of 63 kDa, with a pI of between 5.8 and 6.0 (Van den Heuvel *et al.*, 1994). Despite being the most abundantly produced endosymbiont protein (Van den Heuvel *et al.*, 1994), it was not possible to locate symbionin on the second dimension 2DE gels in the current study. However, 1DE of whole *M. persicae*, alongside remnants and guts, showed the presence of a strong band in whole aphids of around 61 kDa which was much weaker in remnants and undetectable in guts (Figure 3.1). The dissection technique used to isolate samples from whole aphids removes a large part of the haemolymph and all of the reproductive tissues, eliminating most, if not all, of the endosymbionts and symbionin from the experimental samples. The coincident removal of the majority of endosymbionts and their products, in particular from remnant samples, would be consistent with the small amount of protein seen in this tissue after 1DE (Figure 3.1, tracks 3 and 4; ☆).

### 3.3 Discussion

In this study we have used SDS-PAGE and silver staining to examine the protein composition of aphids. In comparison with other techniques which sample only a small part of the available information, such as enzyme studies, the highly sensitive technique of 2DE has allowed the simultaneous detection of large numbers of aphid proteins. Using this method over 150 proteins from aphid remnants and over 50 from guts were detected, giving a combined total of more than 200 proteins. A previous study of *M. persicae* visualised less than 90 proteins in whole aphid homogenates after 2DE, using the same IEF pH range as in the work carried out during this thesis (see Section 2.2.7.2) (Van den Heuvel *et al.*, 1994). The smaller number of proteins resolved in the study by Van den Heuvel *et al.* (1994) was probably because the relatively insensitive technique of Coomassie blue staining was used.

When the proteins from remnants are compared, the results indicate that, regardless of host plant, over half of the major proteins are conserved across the species examined (Table 3.1). These may consist of structural proteins or proteins with common functions throughout insects, such as house-keeping enzymes and exoskeleton components, as well as aphid specific proteins. In gut tissues more variation in protein composition was observed between species (Table 3.3), and while protein conservation was found, it was at a much reduced level compared to that in remnant tissues. What could the reasons be for finding such different amounts of protein variation between the two types of tissue, at the species level?

In an examination of intra-specific variation in mice using 2DE analysis of tissue proteins (Klose and Feller, 1981), a higher level of genetic variation in liver

proteins was found compared to those of brain tissue. It was proposed that there could be two explanations for this: (1) the genes encoding proteins from brain tissue are actively constrained from mutation, as only conserved proteins can carry out the correct functions, but liver proteins are not under the same level of constraint; or (2) there are no constraints on any tissues, but the liver tissue is positively selected to produce variation when challenged by environmental stimuli. As the current literature concerning the effects of plant derived compounds on insects is examined (e.g. Jongsma and Boulter, 1997) it becomes evident that a selective process which produces variation is likely to be operating in insect gut tissue, as a response to diet. Konarev (1996) has suggested that, as a consequence of plants producing defensive chemicals, insects have evolved digestive mechanisms to overcome them. Therefore, in response to plant factors in their diets, the variation in gut proteins between aphid species observed in the current study would be consistent with gut tissues experiencing selective pressures. This would lead to the production of modified gut proteins to help adapt to substances in their diet and would also mean that each aphid species has evolved different molecules adapted to their particular requirements. As each aphid has a different host plant range, this would also mean that aphid gut proteins are in a state of divergence, rather than convergence, between the aphid species. It is noticeable that in the guts, the most abundant proteins are also those which vary extensively, while the most abundant in the remnants are the most conserved. This could indicate that the variant gut proteins are expressed at high levels because they have an important function in the digestive tract, while variant proteins have minor functions in remnant tissues.

The patterns of proteins identified were also be used to examine the relationships between the various aphid species. All six species of aphid analysed by 2DE in this

study are pests in the UK and four were maintained on the same type of host plant. The analysis of remnants and gut proteins found very different levels of protein conservation and variation in the tissues and a parsimonious analysis of both data sets produced different relationships between the species examined (Figure 3.5 and 3.9). However, neither analysis grouped the aphid species according to their host plants.

The relationships between the species, constructed after analysis of remnant data, were not distinct (Figure 3.5). One of the reasons for the low significance of these bootstrap values could be that the level of protein variation on which the analysis was based was very small (see Tables 3.1 and 3.2) and differences between the species were not sufficient to withstand data shuffling.

The relationships produced after similar analysis of the gut protein data, however, showed strong groupings which agreed with the classification of Heie (1980). The fact that an analysis of the gut protein data reflects the phylogenetic relationships suggests that common proteins have not occurred due to convergent evolution while feeding on the same host plant, but rather are present due to common ancestry.

Further evidence for divergent rather than convergent evolution of aphid gut proteins can be gathered from the differences between the four aphid species raised on the same host plant. While aphid gut proteins appear to be the subject of high selection pressure and therefore are unlikely to be evolving at a neutral rate, the observed variations in gut protein profiles between these species, and those feeding on other host plants, do not alter the expected phylogenetic relationships (Heie, 1980). Species which appear closely related by parsimonious analysis of the protein data are likely to have diverged more recently, thereby retaining more commonality in the profile of gut polypeptides. The presence of a common set of proteins among related aphids may help

to explain the separation of the Macrosiphini examined in this study. Two aphid species (*Ma. euphorbiae* and *A. idaei*), which have very different host ranges and lifestyles (polyphagy and seasonal host alteration versus monophagy), should be closely grouped after parsimony analysis of gut data (Figure 3.9). The implication would be that *Ma. euphorbiae* and *A. idaei* were closely linked species modifying a more related set of proteins than other members of the Macrosiphini. Furthermore, the separation of *M. persicae* and *M. antirrhinii* into a distinct group from the other members of the Macrosiphini is interesting, as these two aphids are known to be very closely related (Blackman and Paterson, 1986). These aphids may only have a distant connection with other Macrosiphini, or the *Myzus* aphids may even belong to some other tribe, such as those described in other aphid classifications (for a review see Ilharco and Van Harten, 1987).

## Chapter 4

### 4 Host Plant Induced Changes In Aphid Gut Proteins

#### 4.1 Introduction

The research described in Chapter 3 found high levels of variation in the protein constituents of gut tissue in different aphid species. The variation seen in these proteins could not be attributed to contaminating host plant proteins, as differences in gut proteins were found in four aphid species fed on the same host plant. However, some aphids have the ability to use a range of host plants and with this type of feeding the aphids are exposed to different dietary components. This Chapter examines the gut protein composition of clones of *M. persicae* and *Ma. euphorbiae*, two polyphagous aphids whose host ranges have some overlap, feeding on two different host plant species.

It has been shown that moving aphids between suitable host plants initially reduced aphid fecundity, but full reproductive potential was eventually recovered after feeding on the new host for a time (Douglas, 1997; Wool *et al.*, 1995). This indicated that there was a period of adaptation, by the aphids, to the new host. In the case of an aphid clone, because the genotype is fixed (see Section 1.2.2), this would require some form of internal adaptation. The most likely location for any adaptive response would be the aphid gut, which may react to environmental challenge, such as changes in the nutrient status of different plant species, with the production of new proteins, possibly

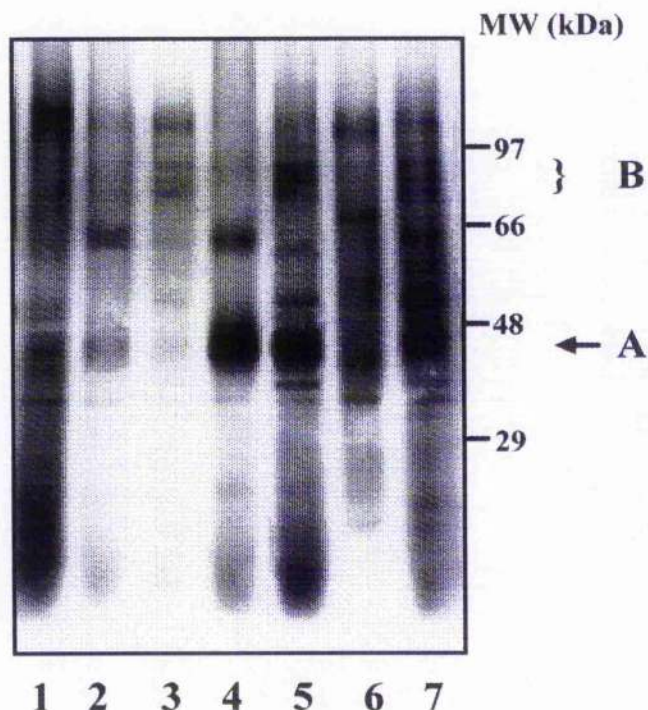
enzymes (Egaas *et al.*, 1991). If the response of the aphid to dietary components includes changes in the gut proteins there may be implications for control methods directed at the gut. For example, if the response to dietary factors involves switching on gut proteases, a control method such as the targeting of specific gut proteins with antibodies or antibody fragments, could be jeopardised as the proteases might break down the antibody molecules. (See also Chapter 6, Section 6.3.3.). Furthermore, if the response to stimulation were to include production of one protein instead of another, gut targets could be lost with a concomitant loss of aphid control. It was therefore very important to characterise the protein composition of an aphid gut when the aphids were fed on different plant types, and to establish if any changes were occurring.

## 4.2 Results

### 4.2.1 One Dimensional Electrophoresis Of Gut Proteins From *M. persicae* On Different Crop Plants

Due to small sample sizes, initial characterisation of the gut proteins from field collected aphids were carried out using 1DE. The studies of field collected and cultured *M. persicae* both indicated that different proteins were being found in the guts of samples feeding on different host plants (Figure 4.1). Those *M. persicae* which had been collected from, or were maintained on, brassica species showed some differences in protein banding patterns from *M. persicae* sampled from, or cultured on, potato. For example, 3 bands between 70-90 kDa were noted in the gut proteins from *M. persicae* feeding on potato (Figure 4.1. Lanes 3, 5 and 7). In contrast, bands of around 40 kDa





**Figure 4.1**

**1DE comparison of gut proteins from *M. persicae* collected from different host plants.** Each sample contained eight guts and proteins were separated using 1DE followed by visualisation with silver staining. Lane 1, *B. brassica* (host plant- Brussels sprouts); 2, *M. persicae* (host plant - turnip); 3, *M. persicae* (host plant- potato); 4, *M. persicae* (host plant- Chinese cabbage); 5, *M. persicae* (host plant- potato); 6, *A. idaei* (host plant- raspberry); 7, *M. persicae* (host plant- potato). A large band of approximately 40 kDa was found in the gut proteins of *M. persicae* fed on brassicas, (indicated as protein A). A second group of proteins, of approximately 70 to 90 kDa, were found only in the potato fed *M. persicae*, indicated as protein B.

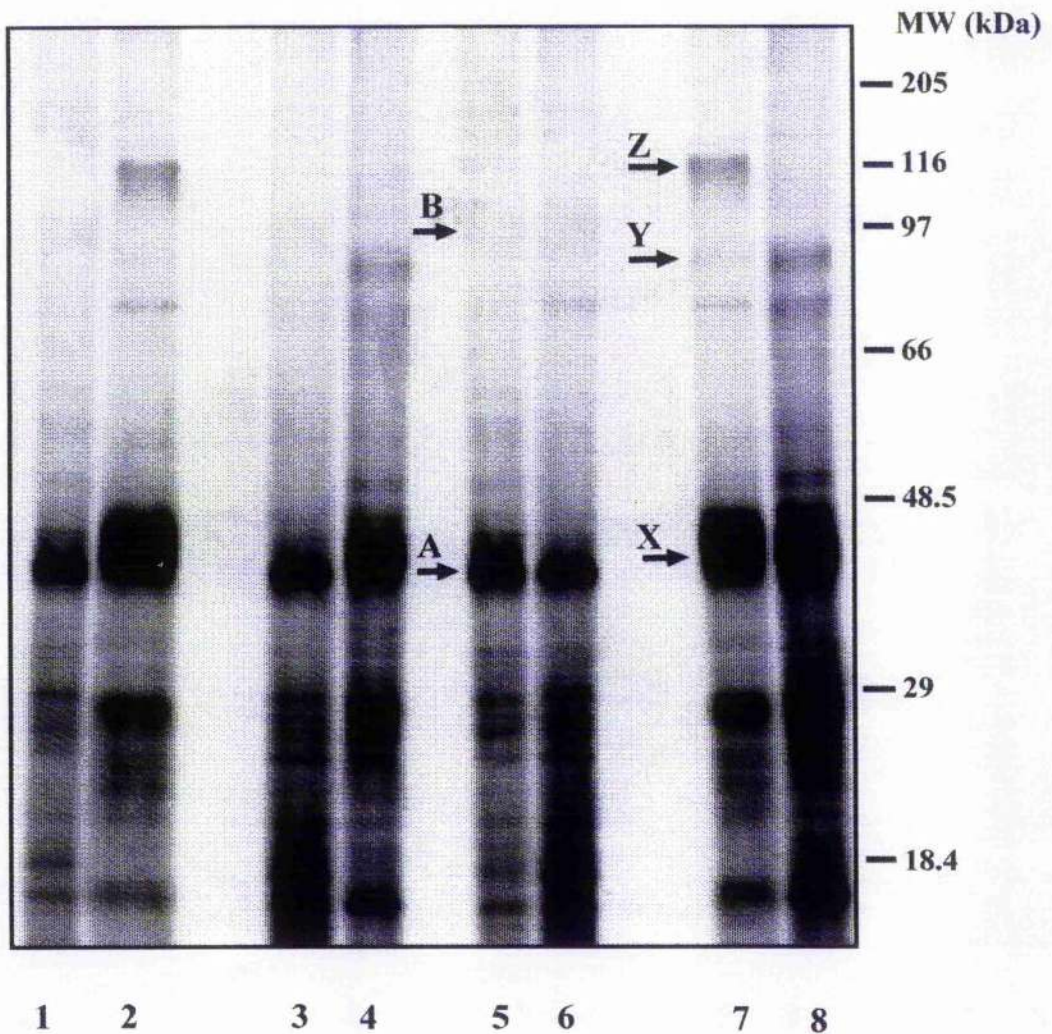
were found in the gut proteins from *M. persicae* which had been feeding on brassicas. While intra-specific variation of allozymes in *M. persicae* is known to be almost non-existent (Brookes and Loxdale, 1987; Wöhrmann and Tomiuk, 1989) there was still a formal possibility that underlying genetic variation could have had some effect on the variation in the observed protein patterns.

Work carried out on *M. persicae* using host plant preference appeared to find some genetic component in the ability to use certain plants as hosts (Weber, 1985). However, more recent research using rDNA fingerprinting of *M. persicae* collected from local potato and brassica crops found a range of genotypes, none of which were associated with a particular host plant (Fenton *et al.*, 1998). Two of the *M. persicae* clones used as samples in Figure 4.1 (Lanes 2 and 7) have been rDNA fingerprinted and are known to be of different genotypes (Fenton *et al.*, 1998). Certain genotypes may thus have become accidentally associated with particular hosts during the production of aphid clones for the current experiment, giving rise to the observed within species protein variation in response to host plant. Therefore, to eliminate any confounding influence of genotype, aphid clones from *M. persicae* and *Ma. euphorbiae* were used. The same genotypes from each species were fed on potato and Chinese cabbage, and the gut protein profiles produced after feeding on these two host plants were compared within the clones.

#### 4.2.2 One Dimensional Electrophoresis Of Gut Proteins From *M. persicae* And *Ma. euphorbiae* Fed On Different Crop Plants

In order to fully assess aphid gut responses to different host plants it was





**Figure 4.2**

**1DE analysis of gut proteins from two aphid species feeding on two different host plants.** Each sample contained eight guts and, after separation by 1DE, proteins were visualised with silver staining. Lane 1; *M. persicae* (host plant- Chinese cabbage); 2, *Ma. euphorbiae* (host plant- Chinese cabbage); 3, *M. persicae* (host plant- potato); 4, *Ma. euphorbiae* (host plant - potato); 5, *M. persicae* ( host plant- Chinese cabbage); 6, *M. persicae* (host plant- potato); 7, *Ma. euphorbiae* (host plant- Chinese cabbage); 8, *Ma. euphorbiae* (host plant- potato). A protein, or proteins, of approximately 40 kDa (indicated as A) were found to vary according to host plant in clonal *M. persicae*, with either a larger amount of a single protein or more proteins in the Chinese cabbage fed aphids. Proteins of similar molecular weight (indicated as X) were found to vary in the *Ma. euphorbiae* clone, but with a greater amount of a single protein or more proteins found in the potato fed aphids. The location of a group of proteins previously identified as being present in potato fed *M. persicae* (Figure 4.1, group B) is marked as B, although the bands are not as clear. Proteins Z and Y are found only in *Ma. euphorbiae* fed on Chinese cabbage and potato respectively.

important to exclude contaminating plant proteins as the source of variation. Therefore, it was necessary to establish that any protein variation seen when an aphid clone was fed on different host plants was of aphid origin. The simplest way to do this was to use clones from two aphid species, feeding on the same host plants. This method would help to identify plant contaminants, as these would appear as the same patterns in both aphid species feeding on the same host plant.

Initially, two polyphagous aphids, *M. persicae* and *Ma. euphorbiae* were used to produce clonal lines in culture on potato. Clones from the two aphid species were maintained in long term culture on potato and samples from each were transferred on to Chinese cabbage. However, even though brassicas are within the host range of *Ma. euphorbiae*, only one clone out of eight initially established on Chinese cabbage survived.

After 5 weeks feeding on Chinese cabbage, guts were dissected from both aphid species and prepared for 1DE. Guts from the same aphid clones fed on potato were also prepared. The samples were examined by one dimensional separation and silver staining (Figure 4.2). Tracks 1 and 2 show gut proteins from both species fed on Chinese cabbage. There were very few bands shared between the species, and consequently very few that might be of host origin. In a similar vein, tracks 3 and 4 are gut samples from both species feeding on potato. Once again there appeared to be a small number of proteins which were common to the two samples and which could therefore be attributed to the host plant. However, the protein bands that varied within each species after dietary change were not those found to be common to both aphid species. Therefore, from these comparisons it is possible to conclude that there are no major detectable proteins from the plant in aphid guts.

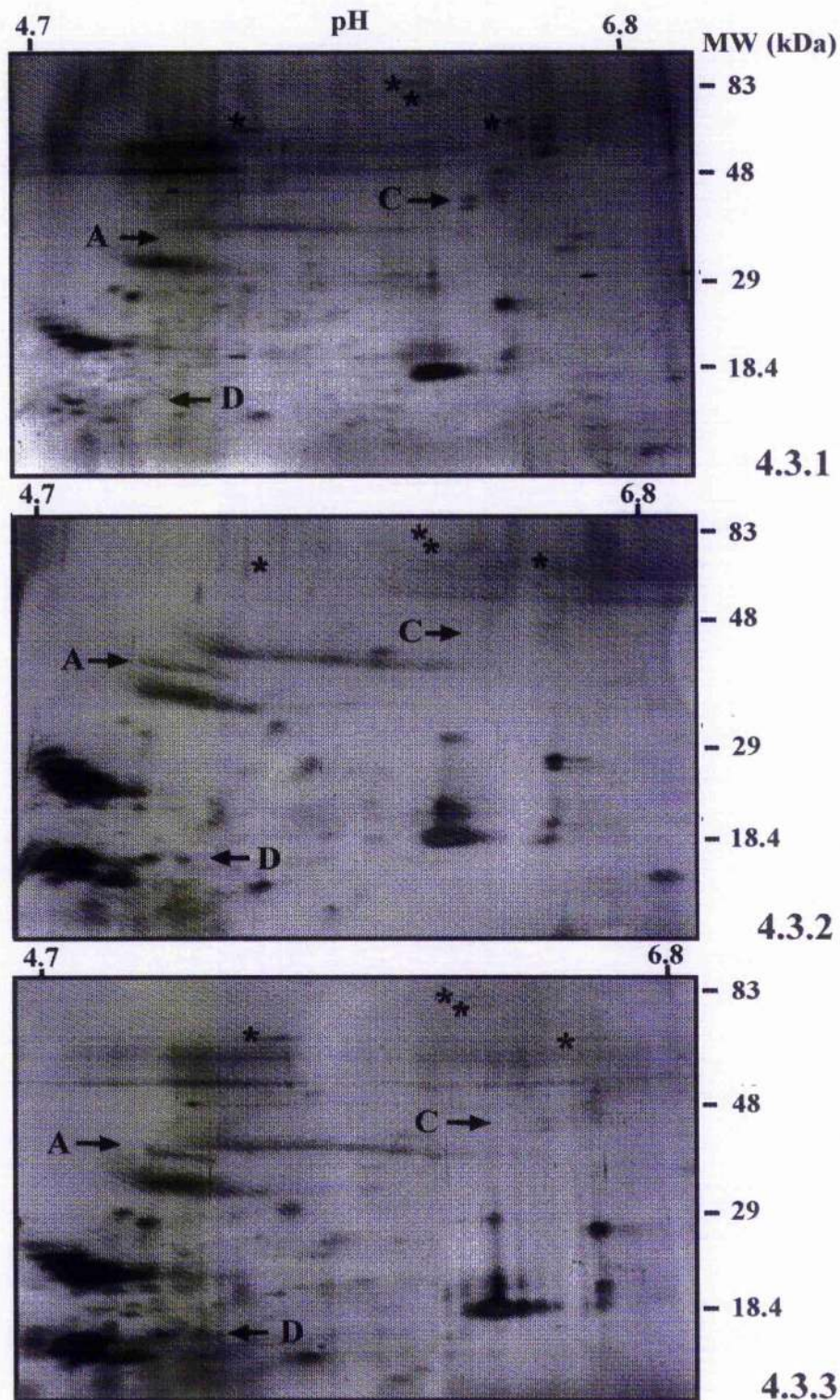
The *M. persicae* gut samples separated in tracks 5 and 6 (Figure 4.2) represent the genetic control of the experimental results in Figure 4.1, with a clonal line replacing the potential genetic diversity of field collected samples. Variations were observed in the gut protein profile of the clone in association with different host plants. One of these differences (Figure 4.2, A) is a group of bands, of between 40 and 45 kDa, which contains a larger number of proteins, or proteins which may be more heavily glycosylated, in aphids fed on Chinese cabbage than in the guts of aphids fed on potato. The presence of three bands of approximately 70-90 kDa seen in the gut samples from field collected *M. persicae* feeding on potato (Figure 4.1, group B) were not as clearly defined in this instance (Figure 4.2, B).

There were several differences in the observed protein profile for the *Ma. euphorbiae* clone on different host plants. One of these difference was again seen in the group of proteins between 40 and 48 kDa (Figure 4.2, X), where a larger number of proteins appeared to be present in the potato fed aphids than the Chinese cabbage fed ones. Other *Ma. euphorbiae* proteins which differed according to host plant were Z (present only in Chinese cabbage fed *Ma. euphorbiae*) and Y (found only in potato fed *Ma. euphorbiae*).

#### 4.2.3 Two Dimensional Electrophoresis And Silver Staining

Having established that host plant material was not a major contaminant of gut proteins and that proteins varied within a clone depending on the diet, an assessment of the variation found within an aphid species was continued with the use of the more sensitive separation method of 2DE, followed by silver staining.





**Figure 4.3**

**2DE analysis of gut proteins from *M. persicae* feeding on two different host plants.** The samples used were as follows:- 4.3.1, guts from potato fed aphids; 4.3.2, guts from Chinese cabbage fed aphids; 4.3.3, mixture containing equal amounts of guts from potato and Chinese cabbage fed aphids. The groups of proteins indicated vary with host plant. Groups A and D are found only in Chinese cabbage fed aphids, while groups B and C are present only in potato fed aphids. The proteins in Group B are marked with an asterisk (\*).

Unfortunately, at this juncture the *Ma. euphorbiae* clone on Chinese cabbage died out. Repeated attempts to re-establish this clone, or one of eight other clones established on potato, on any of the available brassica species failed. As a consequence of time constraints, the experiment proceeded with a clonal line of *M. persicae* cultured on potato and Chinese cabbage.

After 5 weeks feeding on Chinese cabbage, the proteins present in dissected guts from the *M. persicae* clone were examined by separation using 2DE (Figure 4.3.2). Gut samples from the original clonal *M. persicae* maintained on potato were also analysed (Figure 4.3.1) and included in a mixture of the two sample types (Figure 4.3.3). After silver staining, it became apparent that while there were many gut proteins in common when *M. persicae* fed on either host plant, there were also unique proteins in the aphid guts in each situation (Figure 4.3). For example, group C, containing four protein spots with  $M_r$  between 42 and 55 kDa was only found in potato fed *M. persicae*. In contrast proteins in group D, with an  $M_r$  of approximately 10 kDa (Figure 4.3) were only seen where the aphids had been fed on Chinese cabbage.

One group of gut proteins which had been identified on 1DE (Figure 4.2, A) as appearing to alter within the *M. persicae* clone depending on the host plant, had also been shown to vary between species (Figure 3.7, spots 331-348). From the 1DE analysis of gut proteins it was not possible to tell if the higher intensity of staining observed was possibly due to larger numbers of proteins or even whether the amount of protein glycosylation had changed, as a result of differences in host utilisation. However, after 2DE and silver staining, an extra protein was observed in the guts of aphids feeding on Chinese cabbage (Figure 4.3, A). In order to obtain more evidence for the presence of an additional protein within this group (Figure 4.3, A) further protein

characterisation, described in the following sections, was carried out.

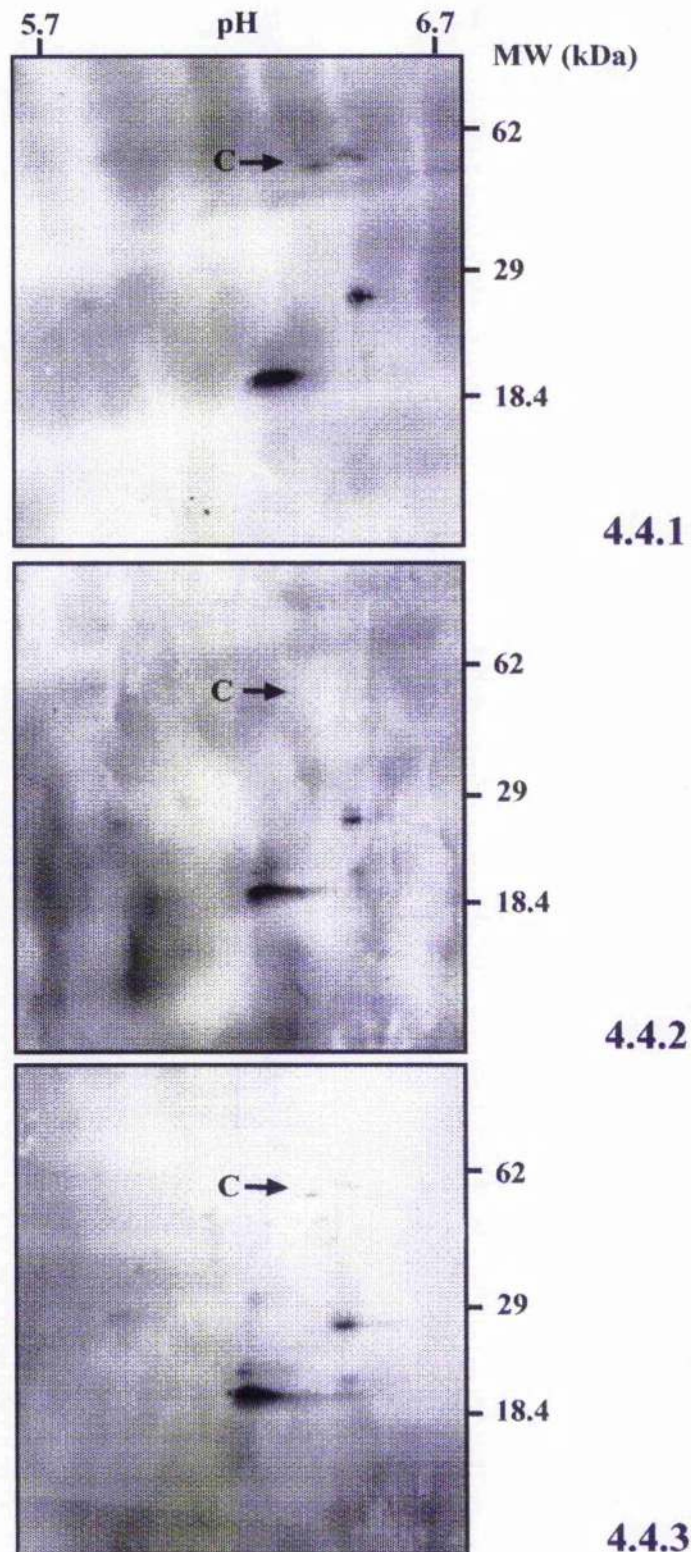
When the *M. persicae* clone was first analysed feeding on potato (Figure 4.2), one protein group previously associated with the field collected *M. persicae* from potato (Figure 4.1, B) was not clearly identifiable. After 2DE and silver staining, these proteins (Figure 4.3, group B) were apparent only in the potato fed aphids, confirming this result and the usefulness of 2DE as method of aphid gut protein analysis. However, it was possible that subtle differences in the protein profiles were masked by all the information revealed by silver staining of gels after 2DE. To address this and other problems, Western blotting and probing with selective binding agents, such as lectins and antibodies, was used. This technique would help to identify subsets of the proteins present without a reduction in sensitivity.

#### 4.2.4 2DE And Western Blotting

##### 4.2.4.1 2DE Blots Of Gut Protein Probed With Peanut Agglutinin (PNA)

Lectins are proteins which bind to different sugar residues in the complex secondary structures of glycosyl side chains. The function of plant lectins has not yet been fully elucidated, but some researchers believe that these plant products have an important role in the defence of plants against insects (Czapla, 1997; Gatchouse *et al.*, 1995). Lectins are very useful tools for investigating the number of glycoproteins present in heterogeneous protein samples and in this study they have been exploited to analyse the complex protein profiles of the aphid gut. As lectins are proposed to be plant defence factors which therefore act as selective agents on the gut proteins themselves, binding to any of the gut proteins may indicate that they are under natural





**Figure 4.4**

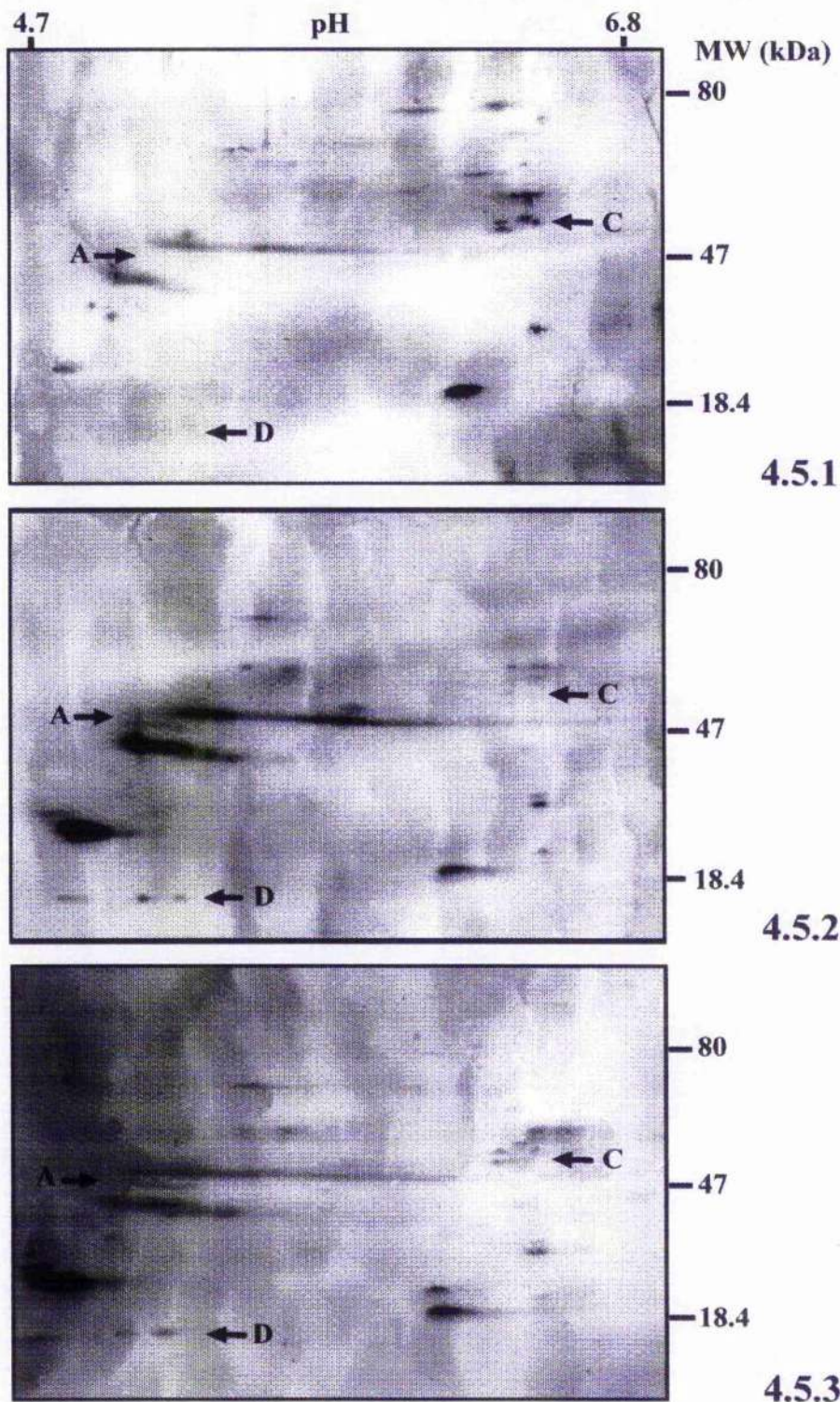
**Pattern of PNA binding to gut proteins from *M. persicae* feeding on two different host plants.** The gut proteins were separated by 2DE and Western blotted before being probed with PNA. The samples used were as follows:- 4.4.1, gut proteins from potato fed aphids; 4.4.2, gut proteins from Chinese cabbage fed aphids; 4.4.3, mixture containing equal amounts of guts from potato and Chinese cabbage fed aphids. The group of proteins marked as C were found only in aphids fed on potato, corresponding with those found on silver staining of equivalent samples (see Figure 4.3).

selection from these plant factors. Probing blotted gut proteins with the lectin peanut agglutinin (PNA), which specifically recognises galactose residues, found that only a small subset of the gut proteins was recognised. A section of this PNA blot containing all the information is shown (Figure 4.4). When the pattern of PNA binding to proteins from potato fed *M. persicae* was compared to that of Chinese cabbage fed *M. persicae*, one difference was observed (Figure 4.4, B). A group of four spots showing PNA binding in the potato fed aphids were absent from the pattern found with the Chinese cabbage fed aphids. All the proteins which displayed binding affinity for PNA, including the four spots found only in the potato fed aphids, could be identified on the corresponding silver stained gels (Figure 4.3).

#### 4.2.4.2 2DE Blots Of Gut Protein Probed With Concanavalin A (Con A)

Concanavalin A is a lectin that recognises  $\alpha$  mannose residues. These residues are found in most glycoproteins as they form part of the core glycan structure. When this lectin was used to probe 2D blots of gut proteins from *M. persicae* fed on the two host plants (Figure 4.5), the subset of gut proteins recognised was much larger than that seen with PNA binding (Figure 4.4). Interestingly, when the proteins bound by Con A were compared to those visualised with silver staining, it could be seen that many of the major groups of proteins found on the silver stained gels were recognised, indicating that these proteins were glycosylated. Indeed, the extent of observed micro-heterogeneity suggests that these were very heavily glycosylated proteins with complex carbohydrate structures, confirming earlier speculation to this effect (see Sections 3.6 and 4.2.3). Once again, differences were observed in the binding of the lectin to gut proteins according to the host plant of the aphid clone. Groups of proteins which varied





**Figure 4.5**

**Pattern of Con A binding to gut proteins from *M. persicae* feeding on two different host plants.** The gut proteins were separated by 2DE and Western blotted, before being probed with Con A. The samples used were as follows:- 4.5.1, gut proteins from potato fed aphids; 4.5.2, gut proteins from Chinese cabbage fed aphids; 4.5.3, mixture containing equal amounts of gut from potato and Chinese cabbage fed aphids. The protein in the centre of group A was only found in aphids fed on Chinese cabbage, as were all the proteins in group D. The proteins in group C, however, were only seen in potato fed aphids.

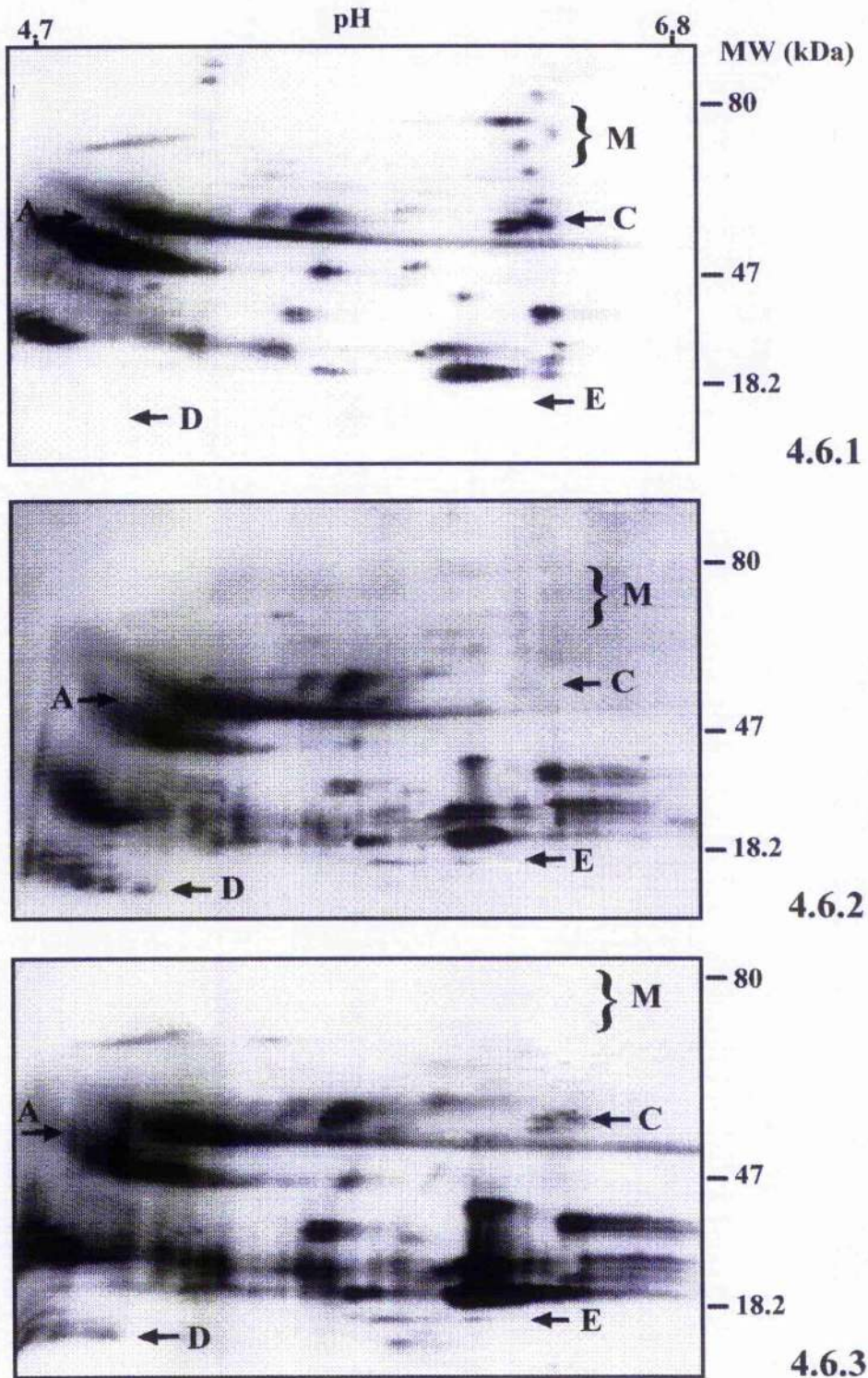
between host plants are indicated in Figure 4.5. Proteins in group D were detected only in aphids fed on Chinese cabbage, as is protein A. The presence of protein A in Chinese cabbage fed aphids probably corresponded to the difference at 40-45 kDa between tracks 5 and 6 in Figure 4.2. This observed variation appeared to be due to the presence of an additional glycoprotein in the gut of the Chinese cabbage fed aphids. Group C were detected only in the potato sample with PNA (Figure 4.4) and this group was also bound by Con A (Figure 4.5).

Aside from proteins which were either present or absent, depending on host plant, there were those which appeared to be the same proteins with slightly different side-chain structures, perhaps due to extra glycosylation residues. This is represented by an extended charge train in the Chinese cabbage pattern (group A, Figure 4.4). These proteins belong to a group that had previously been identified on silver stained 1DE and 2DE gels as an interesting group, varying between aphid species (Figure 4.2, group A; Figure 3.4, spots 331-348).

#### 4.2.4.3 2DE Blots Of Gut Protein Probed With Antibody

Finally, blots of the potato and Chinese cabbage fed *M. persicae* were probed with an antibody raised against the gut proteins of *M. persicae* cultured on Chinese cabbage (Figure 4.6). The overall pattern of antibody binding to the aphid gut proteins was similar, regardless of the source of aphids. However, many of the same groups of proteins which had been identified in the aphid gut in association with a specific host plant using lectin binding, were identified by the anti-gut antibody (Figure 4.6, proteins A, C and D). The antibody also recognised a group of proteins (Figure 4.6, E) present only in the guts of Chinese cabbage fed aphids, which had not been recognised by either





**Figure 4.6**

**Pattern of anti- *M. persicae* antibody binding to gut proteins from a *M. persicae* clone fed on two different host plants.** The samples used were:- 4.6.1, guts from potato fed aphids; 4.6.2, guts from Chinese cabbage fed aphids; 4.6.3, mixture containing equal amounts of guts from potato and Chinese cabbage fed aphids. The groups of proteins indicated vary with host plant. Groups D and E, and the protein in the centre of group A, were only found in aphids fed on Chinese cabbage. Group C was found exclusively in potato fed aphids. The group of proteins indicated as M were not found in all the replicate tests carried out, and it was therefore not possible to attribute them with certainty to the guts of the potato fed aphids.

of the two lectins.

Many of the proteins recognised by Con A, regardless of host plant, were also recognised by the anti-gut antibody. As Con A binds to secondary carbohydrate structures on proteins, this suggests that many of the gut proteins which invoked an immune response for the production of antibodies were highly glycosylated (see Section 5.3).

#### 4.3 Discussion

The results from 1DE analysis of gut proteins from field collected and cultured aphids suggested that there was variation in the proteins present within a species and that this was dependent on the diet of the aphid. These initial findings, however, may have used aphids with diverse genetic backgrounds (Fenton *et al.*, 1998) and it was possible that the observed differences were a result of the genotypes of the aphids examined. Using the same clonal line of aphids on two host plants removed any genotypic variation and confirmed that there were alterations in the protein complement in response to diet. Preliminary results (from 1DE analysis) also indicate that such changes occur in at least two aphid species.

As two aphid species were used initially, a comparison in their protein profiles on the two host plants (Figure 4.2) established that the observed variation associated with diet was not due to contamination of the aphid samples with host plant proteins. This comparison also showed that there were very few major proteins common to the guts of both species, so if plant proteins were present they were not detectable.

The use of 2DE allowed a much more detailed investigation of the changes in the gut protein profile of a *M. persicae* clone occurring with a change in diet. The three distinct probes used on blotted aphid gut proteins each identified a different subset of the proteins which had been visualised using polychromatic silver staining. Within each of the subsets identified, however, differences were observed in the gut proteins which were dependent on the host plant of the aphid. These differences included proteins found only while feeding on potato and those found only after feeding on Chinese cabbage. Some of the proteins observed in the guts of aphids fed on potato were not present after transfer to Chinese cabbage. This means that in addition to the production of new proteins, some of the proteins were no longer present after a change in diet.

The changes observed in aphid gut proteins in response to diet imply that there is some mechanism in place which can alter the gut protein complement both positively and negatively. At the moment it is not known where such a mechanism could be operating, as control could either be transcriptional or post-transcriptional. However, changes in DNA methylation, which apparently prevent the over-expression of esterase genes in the absence of selective pressure, have been documented in aphids (Field *et al.*, 1996). It is also known that restoration of gene expression can occur when selection is reapplied (Hick *et al.*, 1996). Such a mechanism might account for the variation seen in aphid gut proteins under the selective influence of diet.

As some of the proteins which varied with diet reacted with more than one of the probes, it was possible to gain more information about them. For example, those proteins highlighted as group A binding to Con A (Figure 4.5) were also recognised by the anti-gut antibody (Figure 4.6). Thus, the proteins which varied with the aphid host plant were glycosylated and antigenic. Such characteristics may be useful in the

identification of other varying proteins and may aid in understanding more about which proteins are being affected by a change in diet. For example, if differential glycosylation plays a role in the adaptive response to diet, more variation might be identified by probing blotted proteins with other lectins of different binding specificity. Indeed, lectin probing has already shown that a large proportion of the major aphid gut proteins are glycosylated, with an extremely complex glycosylation pattern. However, at present the significance of this is only a matter of speculation (see Section 7.2).

It was interesting to note that many of the *Ma. euphorbiae* clones could not survive on host plants which were supposed to be within the host range of this species. This may be due to host specialists existing as part of the normal range of aphid genotypes within a generally polyphagous population. As the clones were all effectively screened initially for their ability to feed successfully on potato, there may have been an inadvertent selection of clones with a reduced ability to feed on Chinese cabbage or other brassicas. Such host specialists are usually morphologically indistinguishable from other aphids of the same species, and often the only way to diagnose their presence is through host plant feeding trials (Panda and Khush, 1995; Smith, Khan, *et al.*, 1994).

In other polyphagous insects, such as locusts, the ability to feed on many different host plants is always present. The cost of this strategy to these insects is the maintenance of a range of proteins which enable successful feeding of the insects in the presence of a variety of plant defence compounds (Konarev, 1996). For aphids it would appear that the ability to use different host plants successfully, i.e. to maintain the same levels of reproductive performance, is not instantaneous (Douglas, 1997) and that there is a period of change in the proteins in the aphid gut (Egaas *et al.*, 1991). The necessity



of a period of adaptation to diet during which time the production of different gut proteins is triggered may explain the observations of differential performance of some aphids on different host plants. For example, continuous exposure of *Aphis fabae* to a "non-favoured host plant" eventually lead to improved performance on this host plant (Douglas, 1997). It may also be that the gut proteins induced by certain host plants allow aphids to feed as successfully on some other plants without any further change, while feeding on other plants would not be possible without the production of different proteins. For example, De Barro *et al* (1995) found that, over a season, *Sitobion avenae* clones moved from Cocksfoot to wheat, but not generally from wheat to Cocksfoot. This could be explained if the gut proteins induced by feeding on Cocksfoot were compatible with feeding on wheat, but wheat feeding clones had to produce different gut proteins in order to feed on Cocksfoot. However, this situation has recently increased in complexity, as what was believed to be a single species has actually been found to be three closely related species (Sunnucks *et al.*, 1997).

The interesting aspect of apparent specialist feeders within a general population of a polyphagous aphid species is that they may have lost the adaptive response which enables their counterparts to use many host plants. This may have occurred through adaptation to a host plant which has induced such a radical switch in the aphid gut proteins that the aphid has no ability to feed even temporarily on other plants. If this is the case, the mechanisms by which adaptation to dietary factors is turned off could potentially be used to reduce the host range of aphids, thereby controlling them in crops.

## **Chapter 5**

### **5 Characterisation of antisera raised against aphid proteins.**

#### **5.1 Introduction**

One of the aims of this work was to investigate the potential of the 'hidden antigen' approach for control of plant-feeding pests (see Section 1.9). The use of vertebrate-parasite gut antigens to elicit an immune response in host animals, and thereby achieve protection against these parasites, was described earlier (see Section 1.6). The attraction of this method in vertebrates is the ability to target specific parasite antigens, whose location and structure is known, but which are not normally recognised by the vertebrate immune system. Should any breakdown of control occur it is also possible to establish whether there have been any changes in the target pest molecules and to respond with altered antigens for future immunisations, thus recovering control.

The first step to examine whether antibodies against aphid antigens, particularly gut antigens, would produce suitable levels of aphid control was to raise antisera against aphid proteins. In this Chapter, the production and characterisation of the antibodies is described, while the effect of one of the antisera as an aphid control agent is described in Chapter 6.

## 5.2 Results

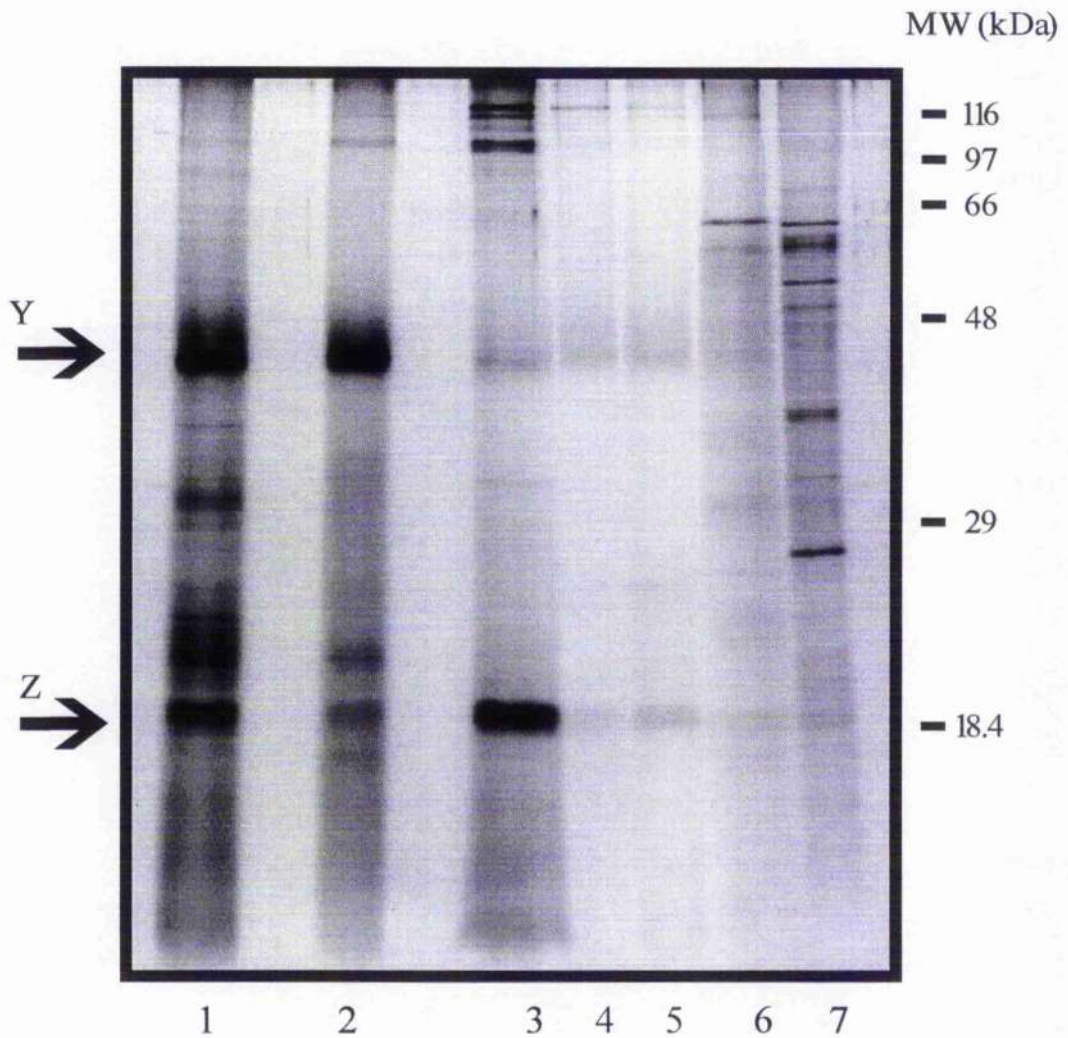
### 5.2.1 Production Of Antibodies To *M. persicae* Proteins

#### 5.2.1.1 Anti-Whole *M. persicae* Antiserum

Adult apterous *M. persicae* feeding on Chinese cabbage were harvested, divided into 500 mg samples and homogenised as described in Section 2.2.3.1. Initially, attempts were made to use detergent extracts of whole aphid homogenates for immunisation (Section 2.2.1.2). However, the amount of protein present in the extracts was highly variable and very low. For example, the concentration of different S1 fractions made from 500 mg of whole aphids varied between 0.13 mg ml<sup>-1</sup> and 0.28 mg ml<sup>-1</sup>. Due to this variation it was decided to use complete homogenates of whole aphids as an immunogen. Whole aphids were homogenised and used to immunise sheep as described in Section 2.2.3.3.

#### 5.2.1.2 Anti-*M. persicae* Gut Antiserum

Guts from adult apterous *M. persicae* feeding on Chinese cabbage were dissected as described in the Section 2.2.3.2, and stored in aliquots of 30, at -70 °C. Detergent extracts were made from 300 aphid guts, as described in Section 2.1.1.2, and samples from these serial extractions were analysed on 1DE (Figure 5.1). The majority of the soluble proteins, likely to be present either in the gut lumen or the gut cell cytosol (Figure 5.1, lane 7; S1) were distinctive from the integral membrane proteins (Figure 5.1, lane 3; S5). Two notable exceptions were proteins of approximately 18.4 and 58 kDa (Figure 5.1, proteins Y and Z). The amount of these two proteins in each extraction increased from S1 to S5 and in the case of protein Y the highest concentration was seen



**Figure 5.1**

**Detergent extracts of aphid gut proteins.** A sample of 300 guts from adult apterous *M. persicae* were subject to serial detergent extraction (see Section 2.2.1.2). The resulting extracts were analysed using 1DE and silver staining. Samples were as follows:- Lane 1, sonicated *M. persicae* guts (starting material); 2, EP; 3, S5; 4, S4; 5, S3; 6, S2; 7, S1. Proteins Y and Z increase in concentration through the serial detergent extractions. S1 contains highly soluble gut proteins, while S5 contains integral membrane proteins.

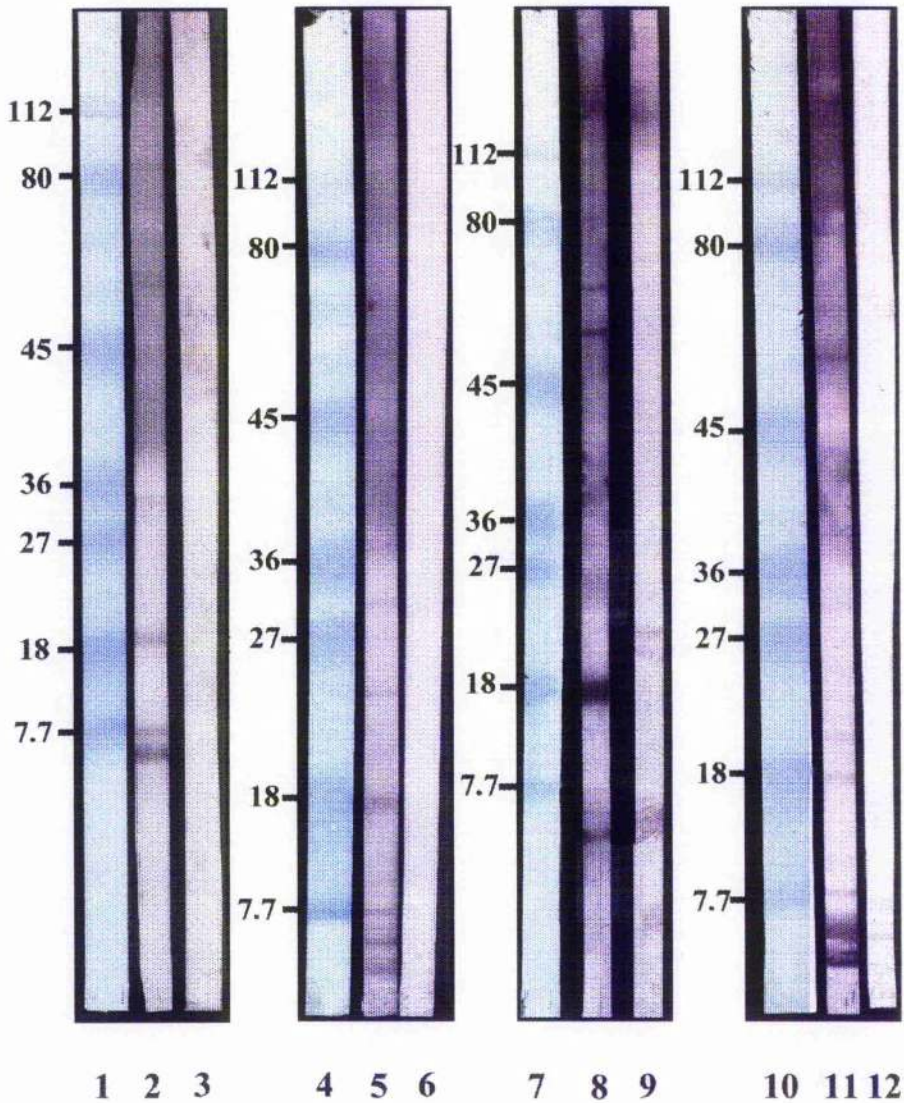
in the end pellet (EP) (Figure 5.1, lane 2; EP). The EP also contained many of the proteins seen in other fractions. Previous experiments which used detergent extracts of *H. contortus* guts as a source of PAs, found that the best responses were achieved when integral membrane fractions (S5) were used as immunogen (Smith, 1993; Smith, Smith, *et al.*, 1994). However, when aphid gut proteins were detergent extracted, the protein content of the S5 fraction was found to be very similar to that of the EP. At each detergent extraction there was an unavoidable decrease in the amount of gut material passed on to the next stage. As dissection of aphid guts was laborious and time-consuming it was not practicable to have such high levels of material loss. Therefore, whole guts were prepared and used for immunisation. It was possible to dissect 40 guts per day, giving 200 per week, and preparation of enough antigen for an entire immunisation procedure took approximately 5 weeks. When sufficient guts had been dissected and isolated (300 guts per immunisation, in a series of at least four injections), they were prepared as described in Section 2.2.3.2, and used for immunisation.

### 5.2.3 Anti-Whole *M. persicae* Antiserum Characterisation

After immunisation with the whole aphid antigen, serial blood samples were taken at intervals of three weeks from the host animal (sheep). Once an immune response had been established, characterisation of the antiserum was undertaken. The antiserum samples produced were analysed for their ability to bind to the total proteins from four aphid species (*M. persicae*, *M. antirrhinii*, *R. padi* and *Ma. euphorbiae*) as shown in Figure 5.2.

The pattern of binding to whole aphid samples showed that large numbers of proteins reacted with the anti-whole *M. persicae* antiserum in all four aphid species





**Figure 5.2**

**Binding patterns of anti-whole *M. persicae* antibodies to proteins from four aphid species.** Aphid proteins from *M. antirrhinii*, *M. persicae*, *Ma. euphorbiae* and *R. padi* were separated on 1DE and Western blotted. Blot strips from each species were either probed with anti-whole *M. persicae* antibodies (strips 2, 5, 8 and 11), or were probed only with the second antibody to assess non-specific binding (strips 3, 6, 9 and 12). Samples used were as follows:- Strips 2 and 3, whole *M. antirrhinii*; 5 and 6, whole *M. persicae*; 8 and 9, whole *Ma. euphorbiae*; 11 and 12, whole *R. padi*. Assessment of molecular weights was made by comparisons with molecular weight standards (strips 1, 4, 7 and 10: molecular weights are indicated in kDa).

examined. As the samples analysed contained the proteins from whole aphids, this pattern of cross-reactivity probably reflected the large numbers of conserved proteins which have been found in all the aphids examined so far, the majority of which were remnant proteins (Table 3.1). As has previously been discussed, these shared proteins are likely to consist mainly of structural proteins, housekeeping enzymes, etc. While many of the proteins which reacted with the antibodies appeared to be common to all the species examined, it was possible to see a few unique bands present for each aphid species. Binding to these proteins may represent cross-reactivity which involves secondary carbohydrate structures on the proteins, such as has been found for the cross-reactivity of antisera raised against proteins from *Trypanosoma cruzi* (Xu and Powell, 1991).

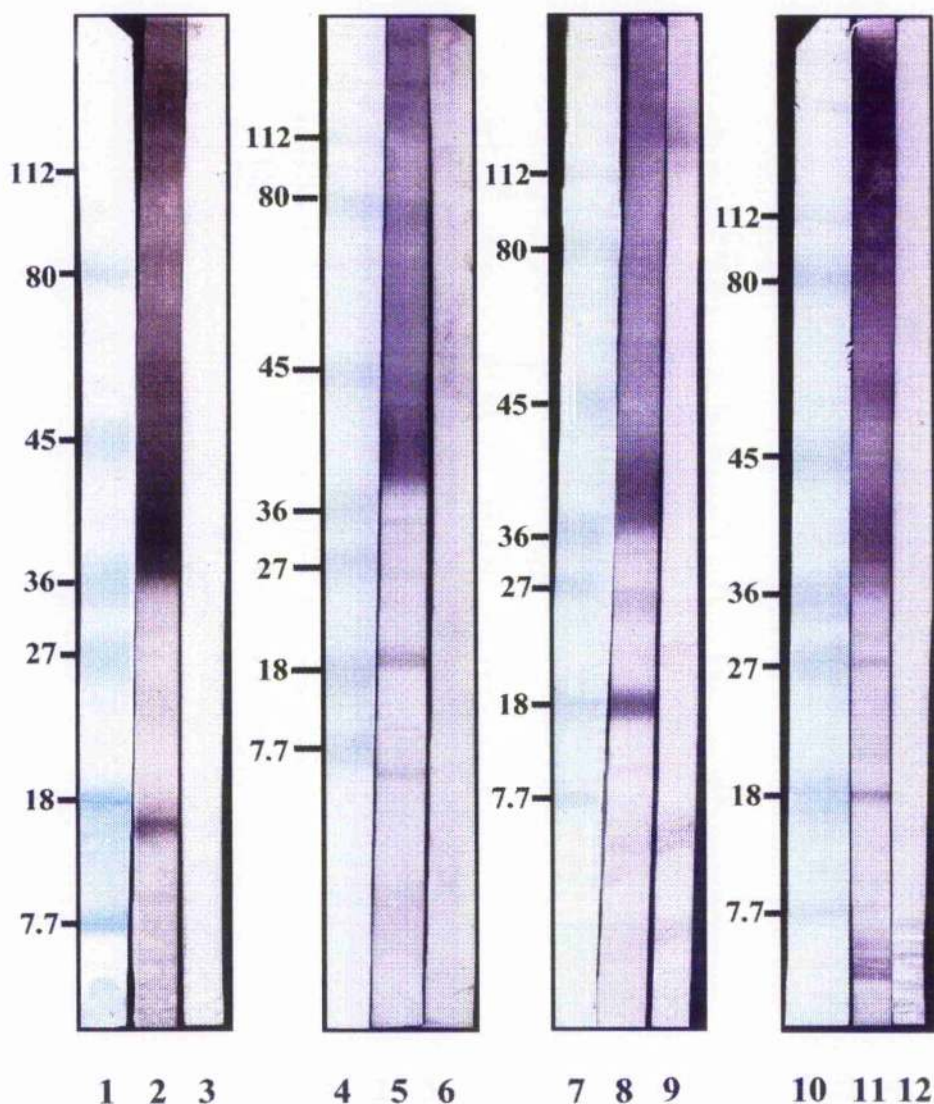
The samples of serum collected at each sampling date for testing of anti-whole *M. persicae* antibodies were very small. Because there were such small volumes of anti-whole *M. persicae* available, further characterisation of the antiserum (for example, by 2DE of aphid proteins and probing of Western blots with the antiserum) was not carried out.

#### 5.2.4 Anti- *M. persicae* Gut Antiserum Characterisation

##### 5.2.4.1 Collection Of Anti- *M. persicae* Gut Antibodies

Samples of the anti- *M. persicae* gut antibodies were obtained following immunisation of the host (sheep), using the same sampling regime as was employed for collection of the anti-whole *M. persicae* antiserum. However, because of the problems encountered with the small samples taken for the anti-whole *M. persicae* antiserum, the sample volume obtained on each occasion was increased to a minimum of 30 ml, and a





**Figure 5.3**

**Binding patterns of anti- *M. persicae* gut antibodies to proteins from four aphid species.** Proteins from *M. antirrhinii*, *M. persicae*, *Ma. euphorbiae* and *R. padi* were separated on 1DE and Western blotted. The samples used were as follows:- strips 2 and 3, whole *M. antirrhinii*; 5 and 6, whole *M. persicae*; 8 and 9, whole *Ma. euphorbiae*; 11 and 12, whole *R. padi*. Blot strips were probed with either anti- *M. persicae* gut antibodies (strips 2, 5, 8 and 11), or were probed only with the second antibody to assess non-specific binding (strips 3, 6, 9 and 12). Assessment of molecular weights were made by comparisons with molecular weight standards (strips 1, 4, 7 and 10: molecular weights are indicated in kDa). Large amounts of indistinct antibody binding can be seen above 36 kDa in strips 2, 5, 8 and 11, making antibody binding differences between the species unclear. In contrast, the antibodies recognise a different distinct protein band, of between 15 and 18.5 kDa, in each species.

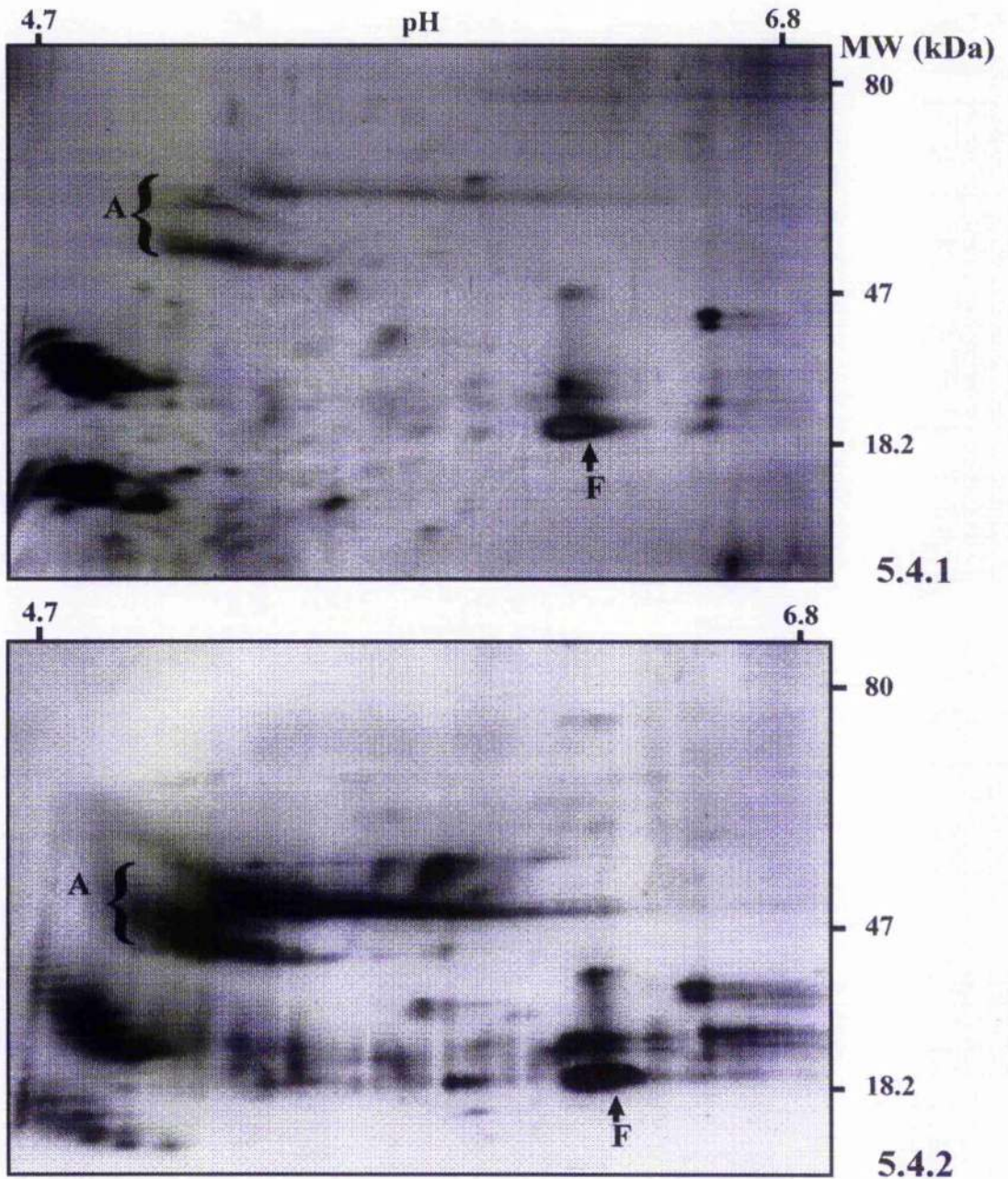


final sample of 500 ml.

#### 5.2.4.2 Anti- *M. persicae* Gut Antiserum Binding To Proteins From Different Aphid

##### Species Using 1DE

When the anti-*M. persicae* gut antibodies was screened for binding to proteins from whole *M. persicae*, *M. antirrhinii*, *R. padi* and *Ma. euphorbiae* separated on 1DE before Western blotting (Figure 5.3), considerable cross-reactivity was seen. It was noticeable that the anti- whole *M. persicae* and the anti-*M. persicae* gut antisera apparently recognised large numbers of the same proteins. As the aphid gut constitutes at least one third of the total aphid, this may not be very surprising. However, a more detailed analysis, such as 2DE, would allow more specific identification of which proteins were being bound. While a greater number of unique proteins were recognised in each of the species than was observed with the anti-whole *M. persicae* antiserum, the lack of resolution seen after one dimensional separation of the proteins made detailed comparison difficult. In general terms, however, the results showed that, despite the differences in composition found on 2DE of aphid guts (Table 3.3), there was a considerable amount of cross reactivity to an antibody which was raised against the proteins present in one species. Interestingly, the anti-*M. persicae* gut antibodies appeared to recognise only one protein of between 14 and 18 kDa in all the species examined (Figure 5.3), but confirmation of the specificity of the antiserum for these proteins would have to be confirmed by further analysis after 2DE and Western blotting (see Section 3.2.3.2).



**Figure 5.4**

**Comparison of total gut proteins and binding of anti- *M. persicae* gut antibodies to *M. persicae* gut proteins.** A sample of sixty guts from adult apterous *M. persicae* was divided in two and each half was subject to protein separation using 2DE. One second dimension gel was silver stained (5.4.1) and the other was Western blotted and probed with an anti-*M. persicae* gut antibody (5.4.2). Proteins which appear to be glycosylated, such as those in group A, and an indistinct area surrounding them, were recognised by anti- *M. persicae* gut antibodies. Gut proteins without apparent glycosylation, such as protein F, were also recognised by the antibodies.

#### 5.2.4.3 Anti- *M. persicae* Gut Antiserum Binding To Proteins From *M. persicae* On

##### 2DE

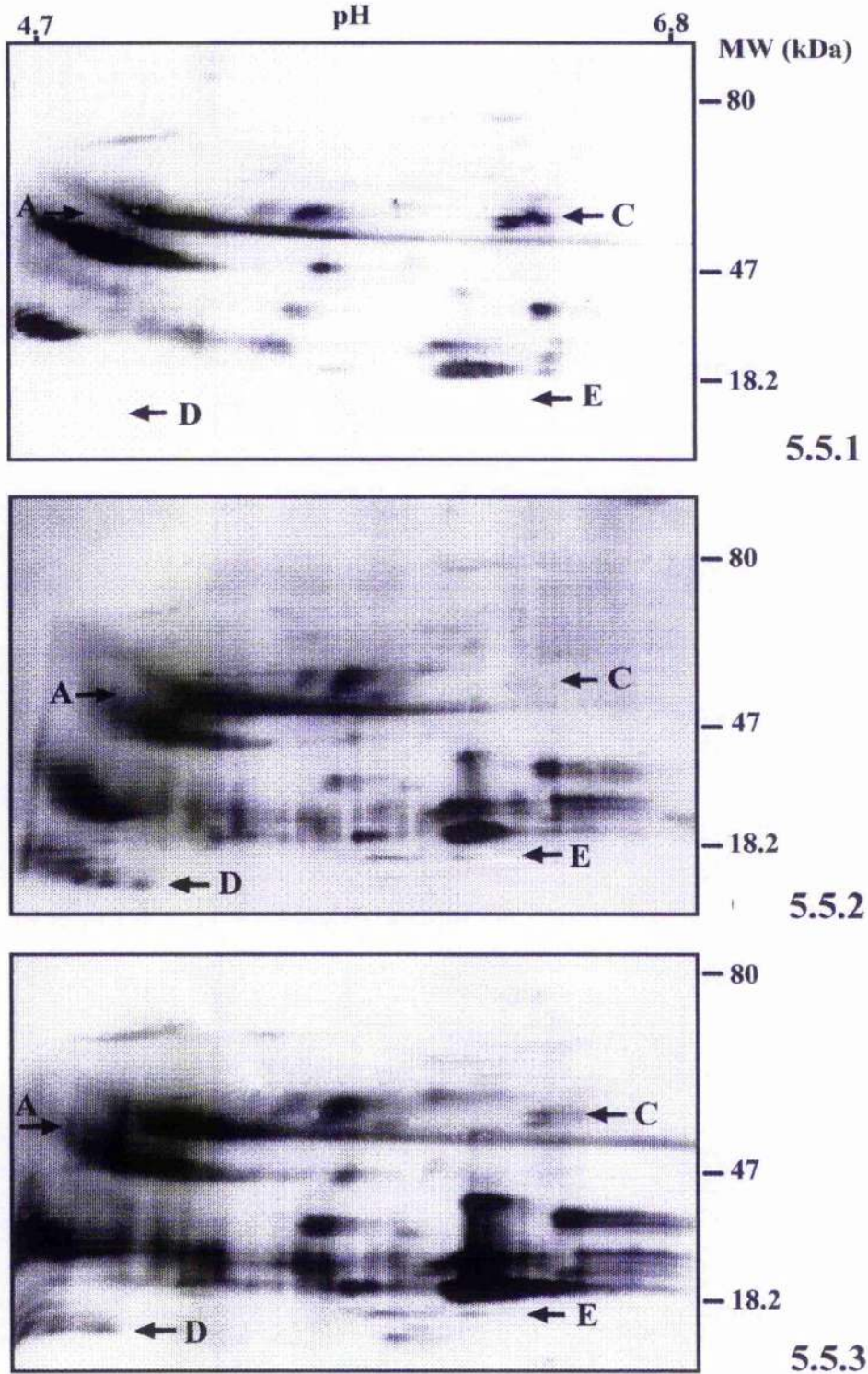
Further characterisation of the anti-*M. persicae* gut polyclonal antibodies was made by binding the antibodies with gut proteins from Chinese cabbage fed *M. persicae* (Figure 5.4). The proteins had been separated by 2DE and Western blotted before being probed with the polyclonal anti-gut antibodies. A comparison between the total protein revealed by silver staining (Figure 5.4.1) and the proteins which were bound by the polyclonal anti- gut antibodies (Figure 5.4.2) showed that many of the gut proteins were recognised by the antiserum and all of the major gut proteins were stained intensely. Of particular interest was the reaction of the antiserum with a large group of proteins (Figure 5.4, group A) which seem to have equivalents in a number of aphid species (Figure 3.7, protein spots 331-348). In *M. persicae*, from a comparison of gut proteins recognised by ConA (Figure 4.5) with those showing anti-gut antibody binding (Figure 5.4) it would appear that group A (Figure 5.4) and several other groups of major gut proteins recognised by the polyclonal antibodies are highly glycosylated. Gut proteins without apparent glycosylation were also recognised by the polyclonal antibodies, such as the protein of about 18 kDa identified after 1DE (Figure 5.3, lane 2; proteins whole *M. persicae*), which can also be seen on 2DE (Figure 5.4, protein F).

#### 5.2.4.4 Anti- *M. persicae* Gut Polyclonal Antibodies Binding To 2DE Separated

##### Proteins From A *M. persicae* Clone Fed On Two Different Host Plants

The gut proteins from clonal *M. persicae* raised on two different host plants (Chinese cabbage and potato) showed slightly different anti- *M. persicae* gut polyclonal antibody binding patterns (Figure 5.5.1 and 5.5.2). An analysis of this antibody binding identified proteins that were only found when the clone had been fed either on Chinese





**Figure 5.5**

**Binding of anti- *M. persicae* gut antibodies to gut proteins from a *M. persicae* clone fed on two different host plants.** The samples used were:- 5.5.1, guts from potato fed aphids; 5.5.2, guts from Chinese cabbage fed aphids; 5.5.3, mixture containing equal amounts of guts from potato and Chinese cabbage fed aphids. The antibodies recognise large numbers of proteins in the aphid gut regardless of the host plant on which the aphids were feeding. Also recognised were groups of proteins that vary with host plant. Groups D and E, and the protein in the centre of group A, were only found in aphids fed on Chinese cabbage, while group C was found exclusively in potato fed aphids. When the clone was fed on either host plant, the anti-gut antibodies recognised both distinct proteins, for example those in groups C, and also proteins plus indistinct areas around them, such as the proteins and area surrounding group A.

cabbage or on potato. For example, proteins in group C were identified in the guts of the potato fed *M. persicae*, but not in those fed on Chinese cabbage. Similarly, proteins in groups A, D and E were observed in aphids fed on Chinese cabbage, but not in those raised on potato. From the work described in Chapter 4, these (specific) differences observed in clonal aphid gut proteins fed in this way have been attributed to changes induced in the aphid gut in response to the chemical composition of the host plant, and not to the presence of contaminating host plant proteins (Figure 4.2).

### 5.3 Discussion

From the work described in previous chapters it would appear that there are detectable differences in aphid proteins between species. These were particularly evident for gut proteins of the different species and protein variation was also found within an aphid clone feeding on different host plants. Therefore, it appeared unlikely that antibodies raised against any one species of aphid, feeding on one particular host plant, would recognise all aphid gut proteins. It is possible that this could have been addressed by raising antibodies to more than one aphid species, however, constraints of time and resources meant that only *M. persicae* was used as antigen source for raising antibodies in sheep. Despite this potential limitation, *M. persicae* proved to be a very useful model plant-pest as it can be successfully raised on several hosts or on artificial diets and it was a reasonable size for the recovery of gut tissue.

In Chapter 3, characterisation of the remnant proteins of aphids found them to be highly conserved between species (Section 3.2.3.1). In view of this observation, the cross-reactivity of an anti-whole *M. persicae* antiserum with proteins from other aphid species is not particularly surprising, as many of the proteins recognised are likely to be common to all the aphid species examined. It is possible that all of the remnant proteins

which react with the antiserum are unique to aphids and are therefore suitable targets for aphid control. However, the high level of protein conservation between species (as defined by 2DE co-electrophoresis, size, shape and colour) and the antibody cross-recognition implies that these proteins have common structures and are therefore likely to fulfil common functions in all the aphid species. If this is the case, there is also an increased chance that they have similar structures and roles in other insect groups and even other eukaryotes, compromising the use of such proteins as targets for aphid specific control strategies. The observed cross-reactivity of the anti-whole *M. persicae* antiserum could also be caused by the antibody recognising secondary carbohydrate structures. Although the analysis of aphid remnant profiles after 2DE (e.g. Figure 3.3) did not reveal large numbers of glycosylated proteins, as assessed by size and shape of the protein spots (Anderson *et al.*, 1985), there was a wide range of apparent glycoproteins observed in the aphid gut (e.g. Figure 3.6). As the antibody was raised against total aphid proteins, any cross-reactivity with secondary carbohydrate structures would therefore be likely to be with gut proteins. This is discussed in more detail in association with the antigut antibody.

The examination of aphid gut proteins in Chapter 3 found high levels of protein variation between the species. While it is biologically very interesting that there is so much variation in the composition of aphid gut proteins, such antigen diversity could be a problem for any antibody-mediated control working across many aphid species. However, when the anti-*M. persicae* gut antiserum was assessed for its binding characteristics, it showed an encouragingly high level of cross-reactivity with the proteins from other aphid species (e.g. Figure 5.3). Of course, binding of an antibody with proteins from other aphid species does not guarantee that the antibody will have the same effect as on the original target species. As described in Section 1.6, cross-

reactive binding of an anti-mosquito antibody to gut proteins of a non-target mosquito did not produce the reduction in fecundity seen after binding in the target mosquito (Ramasamy *et al.*, 1992). To ensure that binding of the anti-aphid gut antibody was going to have the same effects in all species the mechanisms behind the observed cross-reactivity would have to be explored.

One explanation for the observed recognition of proteins in other aphid species by the anti-*M. persicae* gut antiserum could be the presence of protein homologues in the different species. Such homologues would need to have enough physical differences to separate them on electrophoresis, but enough similarities to be recognised by the same antiserum. A case in point would be a group of proteins, present as apparently homologous forms, in a number of the species and identified as spots 331-348, in Figure 3.7. Each species has a slightly different subset of these proteins present in the gut. Variations in this group of proteins have even been found within a *M. persicae* clone when it was fed on different host plants (e.g. Figure 5.5: the protein at the centre of Group A). Such proteins might offer broad spectrum aphicidal targets, as it is more likely that these are molecules which are keyed into the specific requirements of each aphid group, and which will have diverged sufficiently from any ancestral insect progenitor molecules to be aphid specific.

There is also the possibility that some of the proteins present in the gut are precursor molecules. An antibody recognising such a molecule might also bind to the one or more products which are derived from the precursor. For example, an antibody raised against a single aphid salivary protein of 154 kDa showed binding to several aphid proteins, including two smaller salivary proteins of 66 and 69 kDa (Baumann and Baumann, 1995). The two smaller proteins had identical N-terminus sequences, and showed similarities with the N-terminus sequence of the larger protein, suggesting that

they were products of processing of the larger protein. An examination of silver stained gut proteins from Chinese cabbage fed aphids (Figure 5.4.1) shows that a group of proteins in the guts of potato fed aphids which are bound by the anti-gut antiserum (Figure 5.5, group C) are not present. As the antiserum was raised against the guts of aphids fed on Chinese cabbage, the recognition of these proteins could be due to their production from a precursor molecule.

Another possible explanation for the cross-reactivity of the anti-*M. persicae* gut antiserum with proteins from other aphid species is that the antibodies are reacting with secondary carbohydrate structures, and not specifically with the proteins. Large numbers of the proteins recognised in *M. persicae* guts by the anti-*M. persicae* gut antiserum were also bound by the lectin Con A, indicating that these were glycoproteins. Indeed, Con A binding revealed a very complex pattern of glycosylation among gut proteins (e.g. see Figure 4.5). Glycosylation of proteins has been implicated in other instances of antibody cross-reactivity (Willadsen and Billingsley, 1996; Xu and Powell, 1991) and high levels of gut protein glycosylation in other aphids may provide another reason for the levels of antiserum cross-reactivity observed in this instance.

The variation in response by different species of aphid to the addition of lectins to their diets has been examined by several workers (e.g. Rahbé and Febvay, 1992; Sauvion *et al.*, 1996). If the observed cross-reaction of the anti-*M. persicae* antisera is occurring due to the presence of secondary carbohydrate structures, and not to a reaction with specific proteins, this might also explain the different responses of aphid species to lectins. The lectins may be attaching to different proteins in each aphid species, and therefore having very different effects on each species. Differential binding of lectins to the gut proteins of an aphid clone was observed when the clone was fed on two different host plant species (see Chapter 4, Figures 4.4 and 4.5). This shows that even within a



clone the pattern of lectin binding is changeable, which may make lectins a far from ideal aphid control measure.

The reasons for the cross-reactivity seen with both anti-whole *M. persicae* and anti-*M. persicae* gut antibodies have yet to be fully explored. A more extensive investigation of the antibody binding patterns to the 2DE separated proteins of aphid species, other than *M. persicae*, would help to identify any homologues or common precursor molecules. Furthermore, deglycosylation of the gut proteins would help to show how important glycosylation is in antibody binding and begin to elucidate the mechanisms of cross-reactivity. Aside from examining cross-reactivity with other aphid species, it would also be desirable to investigate whether either of the polyclonal antisera will bind to proteins from other insect groups, especially those which feed on aphids, such as lacewings. The nature of any cross-reacting proteins, of both aphid and non-aphid origin, would have to be ascertained to ensure the safety of non-target organisms.

An examination of the gut protein blots in Figure 5.3 shows that some protein spots are intensely stained. The intensity of staining, and therefore binding, of antibodies may reflect the capacity of these proteins to induce an immune response. The antigenic ability of a protein or small subset of proteins may have implications on suitability for their future use as control targets. Where proteins fail to elicit an immune response, it will obviously be impossible to test for any aphicidal quality of antibodies raised against them. Conversely, where a protein is highly antigenic, an overwhelming immune response may mask antibodies to other co-immunising proteins. In the future, fractionation of the immunising antigens will be a step towards solving these problems. After establishing the binding characteristics of the two antisera in the aphid *M. persicae*, they were examined in feeding trials for their effects on aphids (see Chapter 6).

## Chapter 6

### 6 Effects of anti-*M. persicae* antibodies on feeding aphids

#### 6.1 Introduction

At present, the most commonly used method of aphid control is the application of insecticides. However, the recent identification in the UK of highly insecticide resistant *M. persicae*, with both esterase-based and MACE-type resistance (Foster *et al.*, 1998), shows that there is an increasing need to develop alternative control strategies.

In the previous chapter, the production and binding characteristics of the various antibodies raised against *M. persicae* fractions were described. In the present chapter the ability of these antibodies to affect aphid viability was assessed.

The first step to analysing the effects of the antibodies on feeding aphids was the establishment of an artificial diet capable of sustaining *M. persicae* over long periods. There are many artificial diets published for *M. persicae*, both for short and long term culture (for summaries see Kunkel, 1977, and Mittler, 1988). However, it has been noted that, within a species, there are clone specific differences in the ability to survive on any particular diet (Griffiths *et al.*, 1975; Wool and Van Emden, 1981). After three generations on artificial diet (including the initial generation), many clones suddenly die out (Wool and Van Emden, 1981). Therefore, where long term culture of aphids is essential, it is important to have a diet that can sustain the laboratory lines of aphid used. Work previously carried out at SCRI (Nisbet, 1992) used the diet of Griffiths *et al.*

(1975) for the short term feeding of locally collected *M. persicae*. However, when this diet was assessed during the current work for the long-term culture of the locally collected *M. persicae* clone used in host plant trials (Chapter 4) it proved to be unsuitable for maintaining aphids for longer than 5 days. After testing several diets, both oligidic (containing few ingredients with multiple components, such as yeast extract) and chemically defined (containing numerous specific ingredients in known amounts), a modification of the diet from Mittler and Koski (1976) was found to successfully sustain cultures of this *M. persicae* clone for over 10 weeks. The components of the diet are listed in Section 2.2.4. Once the artificial diet had been established, the effects of anti-*M. persicae* antibodies in the aphid diet were assessed.

## 6.2 Methods

Artificial diet (AD) sachets were made up, as described in Section 2.2.4. The various dilutions of control (normal sheep serum: NSS) and immune sera were added to freshly made batches of diet. Where possible, all sachets necessary for an experiment were made at the same time and sachets that were not in immediate use were frozen at -20 °C until required.

Between 4 and 5 adult apterous *M. persicae* were taken from Chinese cabbage and loaded onto each diet sachet with a moistened fine paintbrush. The sachets and aphids were placed into growth cabinets as described in Section 2.2.4. After three days of nymph production, the adult aphids were removed. Nymphs produced in this way were kept on AD until they were adults and then used in experimental testing of the antibodies. Due to problems getting the aphids to move onto sachets by other means,

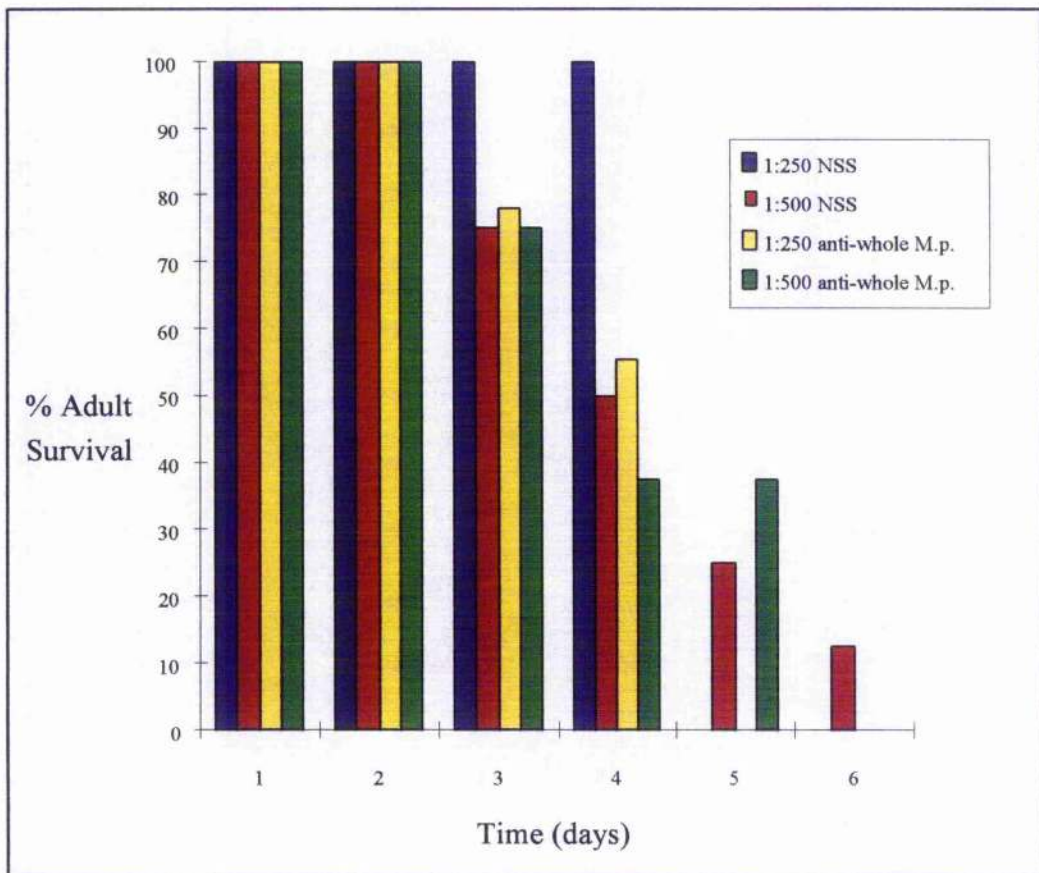
such as inverting the old diet tubes and placing a fresh one on the top for the aphids to walk up onto (Nisbet, 1992), transfer to fresh diet sachets was carried out manually every three days.

### 6.3 Results

#### 6.3.1. Anti-Whole *M. persicae* Antibody

The first antibody to be tested was that which was raised against the whole aphid. Diet sachets containing AD, and AD plus dilutions of control sheep serum (NSS) or anti-whole *M. persicae* serum were prepared. Apterous adults previously fed on AD were loaded onto the test sachets and the effects of the various diets was monitored. However, during the trials using the anti-whole antiserum, many of the sachets containing either normal or immune sera became contaminated with fungi and bacteria. Due to the very high levels of mortality caused by the fungi and bacteria, no clear pattern was obtained from the trials with the anti-whole *M. persicae* antibody (Figure 6.1).

While the method of sachet production was improved by treating the parafilm used to make the sachets with ultra-violet irradiation, because of the problems with contamination and the very small volumes of antiserum available (see Section 5.2.3), no further testing of the anti-whole *M. persicae* antibody was undertaken.



**Figure 6.1**

**Effects of anti-whole *M. persicae* antibodies on feeding aphids.** Apterous adult *M. persicae* previously fed on AD were loaded onto diet sachets at the rate of 20 per sachet. Sachets contained the following:- AD; AD plus normal sheep serum (NSS) at a dilution rate of 1:250 or 1:500; AD plus anti-whole *M. persicae* antiserum (anti-whole M.p.) at a dilution rate of 1:250 or 1:500. All the test treatments were carried out with four replicates and the effects were monitored over 6 days. The data making up this graph can be found in Appendix 1.

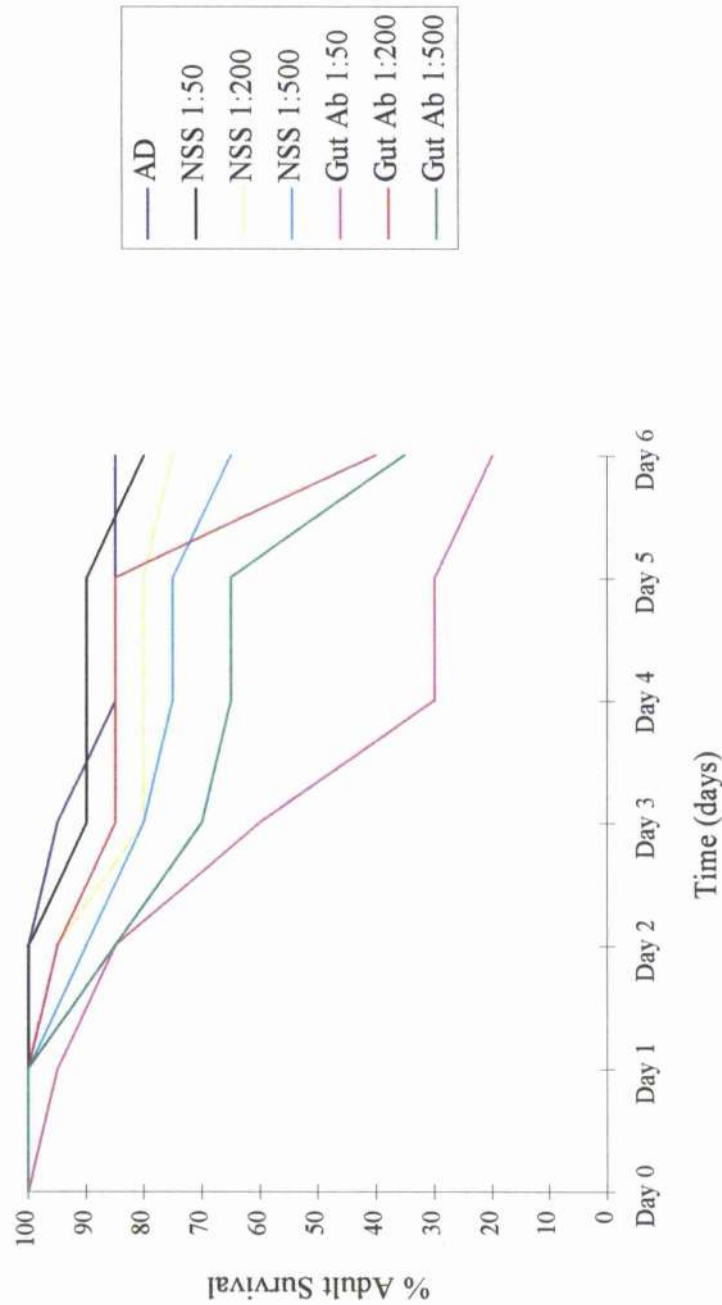
### 6.3.2 Anti-*M. persicae* Gut Antibody

#### 6.3.2.1 Parameters Used To Assess Effect Of Treatment

The second antibody tested in the artificial diet system was raised against the protein present in isolated aphid guts. All the experiments were carried out using the improved methods of sachet sterilisation formulated during testing of the anti- whole *M. persicae* antiserum. After aphids had been introduced onto test sachets containing AD plus normal and immune serum, they were initially monitored for any effects of the treatments on longevity and fecundity. However, the experimental technique was found to cause alate production. Adverse dietary factors (Harrewijn, 1973; 1983) and overcrowding due to rapid population increase (Forrest, 1974) are two of the factors known to trigger the production of alates, but in these experiments it was not possible to differentiate between the two effects. As these alate nymphs were prone to be restless in the diet chambers, yet were unable to move freely, they often died of starvation on the sides of the chambers. This confounded any attempts to measure fecundity, therefore adult survival was chosen as the best performance indicator.

#### 6.3.2.2 Effects Of Antiserum Concentration On Feeding Aphids

The effects of including the anti- *M. persicae* gut antibody in the diet of aphids was initially assessed using a range of antiserum concentrations. The results for a typical feeding trial using three concentrations of NSS and anti-*M. persicae* gut antibody in AD are shown in Figure 6. 2. The trial used adult aphids to avoid the confounding effects of wing production (as described in Section 6.3.2.1), but because of



**Figure 6.2**

**Effects of three concentrations of anti- *M. persicae* gut antibodies on feeding aphids.** Adult apterous *M. persicae* were fed on sachets containing the following solutions: - artificial diet (AD); AD including 1:50, 1:200 and 1:500 concentrations of normal sheep serum (NSS); AD containing 1:50, 1:200 and 1:500 concentrations of anti- *M. persicae* gut antibodies (Gut Ab). The effects of diet on the aphids were monitored in terms of adult survival over 6 days. The data making up this graph can be found in Appendix 1.

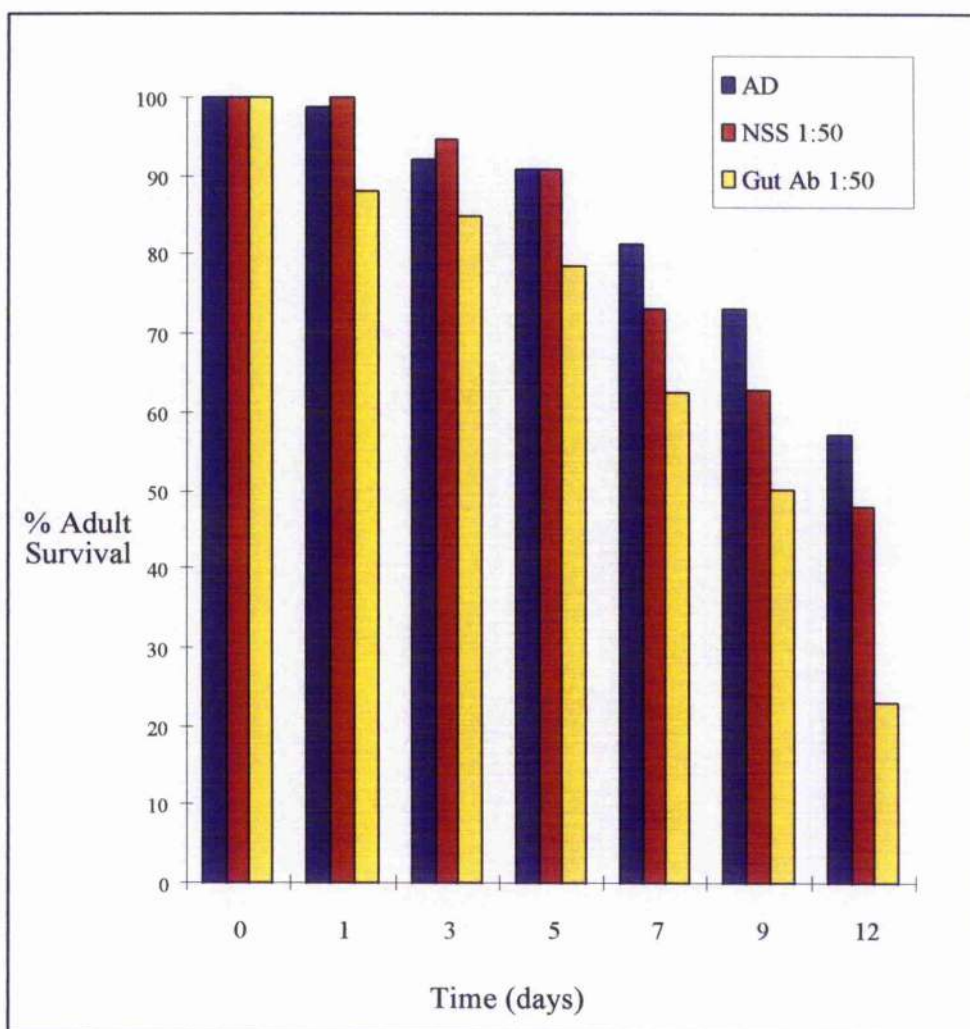
the relatively short life of adult aphids, all of the treatments show mortality. However, the aphids which fed on the diet which included immune serum died at a faster rate. The other two treatments, artificial diet and artificial diet with control serum, gave lower rates of mortality, which were similar to each other. These results suggested that the immune serum was having an adverse effect on aphid survival. As can be seen in Figure 6.2, an anti- *M. persicae* gut antibody concentration of 1:50 in AD gave the fastest mortality rate. To try to reduce confounding effects (see Section 6.4 for discussion) 1:50 was the concentration of antiserum and NSS used in the subsequent feeding trials.

#### 6.3.2.3 Objectivity In Results Of Feeding Trials

Aphid lifesigns were assessed by observing the aphids on the sachets using a stereoscopic microscope. When the aphids feeding on test sachets were examined and the contents of the sachets were known, it was possible that aphid life signs were assessed without objectivity. To eliminate any subjectivity in interpretation of aphid survival, further trials were undertaken. In this case the serum contents of the diet sachets were set up and encoded by a third party. The experiment was then conducted as described in Section 6.3.2.2 and the contents of the sachets was decoded once data analysis was complete. An example of such a trial is given in Figure 6.3. Once again the results indicate that the anti-*M. persicae* gut antibody has a detrimental effect on the survival of feeding aphids, compared to controls.

All the trials carried to date show the same trend for aphid survival on each treatment, i.e. that the total number of aphids surviving on AD plus anti-*M. persicae* gut antibody is lower than the numbers surviving on either base diet or AD plus normal





**Figure 6.3**

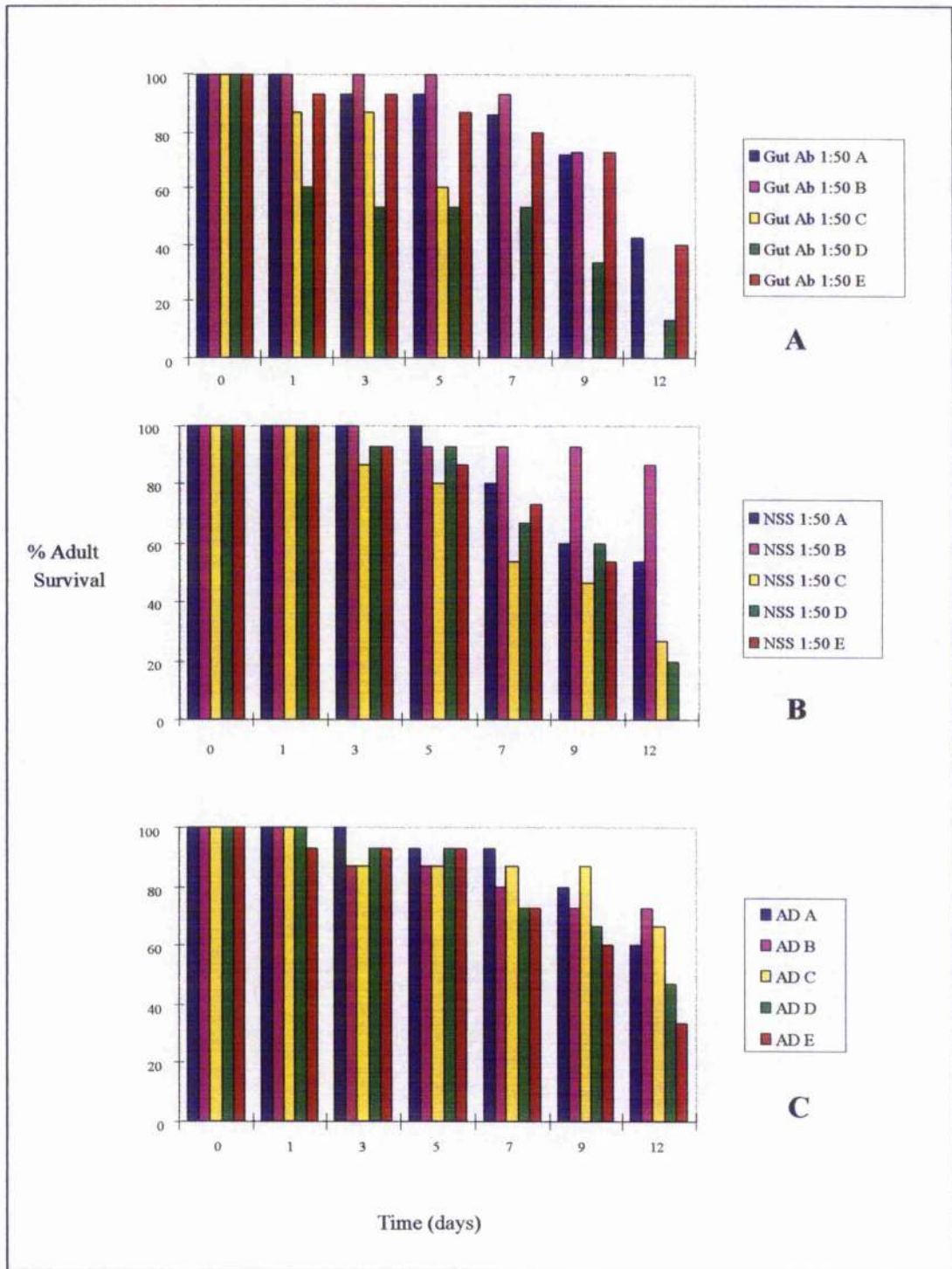
**The effects of anti-*M. persicae* gut antibody on feeding *M. persicae*.** Treatments were as follows: AD, AD including NSS (1:50), AD including anti-*M. persicae* gut antibody (1:50). Each treatment had 5 replicates and the effects of treatment were monitored over 12 days. The contents of sachets were originally identified by a third party using a random code. To maintain objectivity, the results of the experiment were analysed before the identities of the sachets were decoded. The data making up this graph can be found in Appendix 1.

sheep serum. While these results appear to be clear cut, the variation between sachets, within treatments, was considerable. In some cases even the aphids on artificial diet would show high levels of mortality. To illustrate this, Figure 6.4 shows the results from individual sachets before they were collated to produce total adult survival. All the trials carried out with anti-gut antibodies showed such high levels of inter sachet variation across all treatments that there was no statistical significance to the results. Examples of the statistical tests carried out on the data are given in Appendix 1.

### 6.3.3 Movement Of Serum Components From Artificial Diet Into The Aphids

The above results suggested that the anti-*M. persicae* antibody was having an adverse effect on the aphids, but because of the inter-sachet variation described (Figure 6.4) it was not possible to obtain reliable statistical proof of any effect. At a practical level it was just as important to demonstrate that there was actually antibody present within the aphid intestine to cause any of the effects seen. This would provide information both on the quantity of antibody as well as any degradation due to the activity of proteases that may exist in *M. persicae* (Srivastava, 1987).

To assess movement of serum components, from the AD into the aphid gut, aphids fed on diets containing 1:50 dilutions of NSS or anti- *M. Persicae* gut antibody for at least 6 days were carefully dissected so that the abdomens and heads were separated. The two samples produced were prepared as described in Section 2.2.7.1 and the proteins were separated by electrophoresis followed by blotting onto nitro-cellulose (see Section 2.2.7.4). The presence of antibody within these aphid fractions was assessed by reacting the blots with a second antibody that recognised sheep



**Figure 6.4**

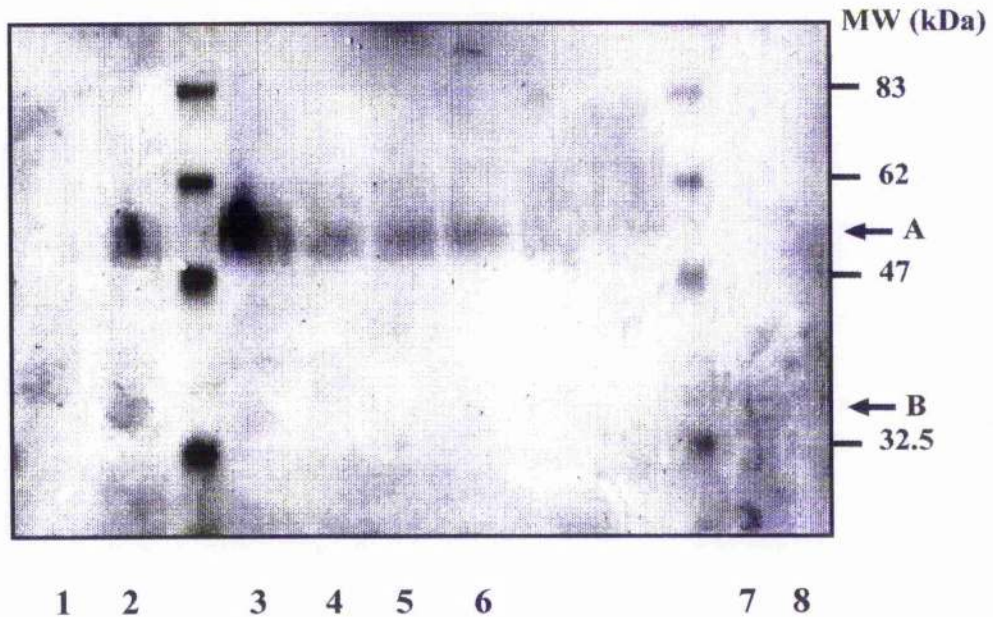
**Inter-sachet variation observed within treatments over one feeding trial.** The treatments used in the trial were: A. AD including anti-*M. persicae* gut antiserum (1:50); B. AD including NSS (1:50); C. AD. Each treatment had 5 replicate sachets (A-E). The effects of treatments on feeding *M. persicae* were assessed over 12 days. The data making up this graph and the statistical analysis of the data can be found in Appendix 1.

immunoglobulins.

This first method of detection did not prove to be sensitive enough to detect any antibody, even in samples of AD containing antiserum, on which the aphids had been feeding. It is possible that in the case of the aphid samples, aphids proteins of the same approximate molecular weight as the IgG heavy and light chains masked any IgG present in the samples by preventing the anti-sheep IgG antibody from binding. An alternative method was used to overcome this potential problem. Protein G is a bacterial protein that has an extremely high affinity for antibody molecules (Björck and Kronvall, 1984; Björck *et al.*, 1987). When Protein G is bound to sepharose beads the combination forms a very effective way of purifying antibodies from complex mixtures (Harlow and Lane, 1988). Therefore, to increase the sensitivity, Protein G was used to affinity purify antibody molecules from a homogenate of the aphid abdomen or head (see Section 2.2.6). The samples of Protein G beads containing the affinity-purified antibodies from aphid samples were added to their own volume of lysis buffer (see Section 2.2.7.1).

The samples were boiled for 4 min and then centrifuged at  $15,800 \times g$  for 4 min. The sample supernatants were analysed by 1DE and Western blotting and probing with anti-sheep IgG antibody (see Section 2.2.7.4). In this case the immunoglobulin bands could clearly be identified (Figure 6.5). Surprisingly, there seemed to be more immunoglobulin in the aphid heads than in their abdomens. This may be due to the antibody encountering a barrier in the head area, such as the oesophageal pumps, from where it passes only slowly to the gut. Encouragingly, the antibody detected in samples of aphid head and abdomens was the same molecular weight as intact heavychain of IgG, suggesting that there was very limited if any proteolytic activity occurring.





**Figure 6.5**

**Passage of IgG into aphids from AD.** Adult apterous *M. persicae* fed on AD containing NSS (1:50), AD containing anti- *M. persicae* gut antibody (1:50), or potato plants, for 6 days were dissected into head and abdomen samples. Using protein G (see Section 2.2.5), proteins isolated from the following samples were separated by 1DE, Western blotted and probed with an anti-sheep IgG antibody to detect the presence of IgG. Lane number: (1) AD; (2) AD containing NSS (1:50); (3) IgG extract from heads of 30 *M. persicae* fed on AD + NSS (1:50); (4) IgG extract from heads of 30 *M. persicae* fed on AD + anti- *M. persicae* gut antibody (1:50); (5) IgG extract from abdomens of 30 *M. persicae* fed on AD + NSS (1:50); (6) IgG extract from abdomens of 30 *M. persicae* fed on AD + anti- *M. persicae* gut antibody (1:50); (7) IgG extract from heads of 30 *M. persicae* fed on potato plants; (8) IgG extract from abdomens of 30 *M. persicae* fed on potato plants. Samples in lane numbers 2-6 show the presence of a strongly reactive protein at the approximate molecular weight of IgG heavy chain (A). Lane 2 also has a weakly reactive band at the approximate molecular weight of IgG light chain (B). (This figure is a composite of two blots. Lanes 1 and 2 with molecular weight standards belonging to one blot and lanes 3 to 8, including molecular weight standards, to a separate blot.)

## 6.4 Discussion

There was a wide variation in response to the ADs tested from the locally collected *M. persicae* clone used in the feeding experiments (see Section 6.1) and finding a diet that was acceptable for the long-term culture of this *M. persicae* clone proved difficult. However, once a diet had been formulated, aphids could be kept as a reproducing colony on AD for at least 10 weeks.

The problems in obtaining a suitable AD for the long-term culture of the locally derived *M. persicae* clone highlighted both the advantages and the disadvantages of the AD system. Synthetic diets are an ideal way to introduce different substances into aphids without the need for further manipulation, such as spraying the aphids with chemicals. All the variables, such as day-length, ambient temperature, humidity, and even genotype of the aphid, can be controlled during the experiments using AD, thus providing unbiased results in which only the introduced dietary factors are monitored. However, by using aphids which can be maintained on artificial diet, a subset of the available genotypes has been selected, which may not be representative of the natural situation or which may actually skew the results towards tolerance or sensitivity to dietary components. When attempting to maintain a broad panel of *M. persicae* with insecticide susceptible and resistant genotypes, it was noticed that the resistant aphids were much less tolerant of artificial diets than susceptible ones (Wool and Van Emden, 1981). The current study used a single genotype to assess the effects of host plant change (Chapter 4) and to examine the effects of anti-aphid antiserum in AD. However, for wider application of the effects of incorporated dietary substances, including the anti- *M. persicae* antiserum, it would be important to try to assess as many genotypes

and species as possible, even though this may not be possible with one generic diet.

In all the feeding trials carried out for the current work there was an apparent deleterious effect of the anti-*M. persicae* gut antibody on adult aphid survival, in comparison to base diet and where normal sheep serum was included in the diet. While this cannot yet be supported statistically, the results are very encouraging and would justify a continued examination of possible antibody mediated control of aphids. The importance of gaining statistical proof of any effect, without resorting to 'data dredging', cannot be underestimated. It is highly desirable that trials should be carried out with large enough numbers of sachets to reduce or remove the inter-sachet variation, which has obscured the trial results to date.

There are, however, at least two alternatives to large-scale trials for reducing the inter-sachet variation. Firstly, the way in which the diets are presented to the aphids may be changed. In an ideal system there would be a continuous supply of fresh diet and a sufficient feeding area that the stress factors, such as the induction of alate production by over-crowding of apterous aphids (Forrest, 1974), were not triggered. If all the test aphids were effectively present on only one of two large diet sachets, a pair-wise comparison could be done, to assess the effects of diet alone. In such a situation it might also be possible to include the extra parameter of nymph production, without the confounding effects of crowding as found on small diet sachets. Systems which can deliver constant fresh diet to fairly large feeding areas have been produced (e.g. Akey and Beck, 1975). However, the manufacture of such systems and large-scale trials were not possible during the limited time and resources available for this study.

The second alternative to much larger experiments is to try to achieve much more rapid and dramatic effects of the anti-*M. persicae* gut antibody on the feeding

aphids, before any sachet to sachet variation has occurred. From the experimental tracking of serum immunoglobulin into the feeding aphid, it would appear that there may be some physical or biochemical barrier preventing the fast passage of molecules, such as immunoglobulins, into the aphid gut lumen. It may be possible to achieve faster action of the antiserum by processing the antibody molecules to produce active fragments, such as those produced after digestion of immunoglobulins with papain or pepsin (Harlow and Lane, 1988). For example, every antibody molecule will produce two Fab fragments, each containing a biologically active antigen-binding domain, after papain digestion (Male *et al.*, 1987). If protein size is a limiting parameter in antibody passage into the gut, then these smaller Fab fragments would pass more freely into the aphid guts and help to shorten the amount of feeding time necessary to produce a deleterious effect.

Whole serum contains a wide range of molecules, only some of which are involved in the immune response (Male *et al.*, 1987). Aside from the production of IgG fragments, it would be prudent to assess the effects of other antiserum fractions or components on feeding aphids. For example, the effects of including antibodies which bind specifically to gut glycoproteins could be assessed by using the lectin Con A to affinity purify glycosylated gut protein, which would in turn be used to affinity purify the anti-*M. persicae* gut antibodies which react specifically with the gut glycoproteins. Some of the current research being carried out on alternative aphid control methods is focused on disruption of gut glycoproteins (for example, Gatehouse *et al.*, 1993; Rahbé and Febvay, 1992) and including this antiserum fraction into AD would help to assess how important glycoproteins are to the aphid.



## Chapter 7

### Final Discussion

#### 7.1 The Variation Of Aphid Proteins Between Species

The work described in Chapter 3 examined the proteins present in six different species of aphid using 2DE and polychromatic silver staining. The number of proteins seen in the remnant fraction of aphids alone exceeded that previously found in studies of total aphid homogenates using other combinations of protein separation and detection methods (Van den Heuvel *et al.*, 1994; Leszczynski *et al.*, 1994; Owusu *et al.*, 1996). As mentioned before (Section 3. 1), only one previous study using 1DE had looked specifically at protein variation between aphid species (Khuda-Bukhsh and Khuda-Bukhsh, 1991). Using homogenates of whole aphids, Khuda-Bukhsh and Khuda-Bukhsh (1991) found some differences between the species examined, but less than thirty proteins were detected in any sample.

Before the current work was undertaken, the only in depth studies of variation in aphid proteins had looked at differences in isozymes and allozymes, marker systems which had been used to give an indication of the relatedness of other eukaryotic species, for example Agromyzidae (Diptera) and Yponomeutidae (Lepidoptera) (Menken & Ulenberg, 1987). When these methods were employed to look at the genetic relationships in aphids, the majority of the studies carried out disclosed little or no variation within aphid species (Brookes & Loxdale, 1987; May and Holbrook 1978; Simon *et al.*, 1995; Suomalainen *et al.*, 1980; Wool *et al.*, 1978). While different aphid

species could be distinguished using allozymes or isozymes (Blackman and Spence, 1992; Khuda-Bukhsh and Khuda-Bukhsh, 1991; Loxdale & Brookes, 1989; Tomiuk & Wöhrmann, 1980), in most cases this still did not represent the large body of variation expected between species.

There are at least two possible explanations for the observed general lack of variation in aphid enzymes within species (Hales *et al.*, 1997). The use of allozymes to give some indication of the rate of genetic change makes the assumption that any mutation occurring will be neutral. Point mutations, deletions and insertions in DNA are considered to be neutral when they leave the function of the encoded protein intact and therefore have no effect on the fitness of the genotype. It is possible that this assumption does not hold for mutations in the enzymes under examination in aphids, perhaps because they have a very specific set of enzyme requirements which reduces the chances that any mutation produced will be neutral (Hales *et al.*, 1997). This would mean that mutations in the genes encoding the enzymes would rarely be tolerated as the majority of such genotypes would be deleterious. In turn, this would produce a false impression of low levels of protein polymorphism.

The second possible cause of low enzyme heterogeneity in aphids is that they have only relatively recently separated into species, and enzymes within the different species have not yet diverged into different alleles (Hales *et al.*, 1997). The recent divergence of aphids into species could also explain the lack of variation found with some other marker systems, such as mtDNA and RAPDs (Al-Aboodi & French-Constant, 1995; Simon *et al.*, 1995; Simon *et al.*, 1996).

Molecular techniques, such as rDNA fingerprinting and microsatellite analysis, which directly examine DNA have been applied both within (Black, 1993; Fenton *et al.*,

1998) and between (Cenis, *et al.*, 1993; De Barro *et al.*, 1995) aphid species. With the information generated by these methods it has been possible to change the view, based on allozyme data, that many aphid species contained very low levels of genetic variation (for example, Brookes & Loxdale, 1987). However, neither the allozyme studies nor investigations using DNA analysis gave any indication about the overall protein complement of aphids. The current study of the molecular characterisation of aphid proteins, using 2DE and silver staining, has revealed a large body of protein variation and protein conservation existing between the aphid species examined, in agreement with the levels of DNA variation found during other research. Aside from gaining a better idea of the total number of proteins in aphids generally, 2DE separation provided good evidence for the existence of protein homologues. Such protein homologues may represent the basic building blocks of aphids, or even insects, from which adaptive proteins are generated. As the silver staining method used also allowed initial assessments of protein quantification (Morrissey, 1981) there is now information on the relative quantities of some of the proteins identified (see Section 3.3). Where enzyme analysis has been undertaken such protein quantification has not been possible as the rate of substrate conversion limits how much staining can occur (Menken and Ulenberg, 1987). Enzyme staining also requires the presence of active enzymes, whereas silver staining does not have such limitations (Morrissey, 1981).

Different levels of protein conservation and variation were observed between remnant and gut tissues for the six aphid species under study and the possible reasons for these different levels of protein change have been discussed (Section 3.3). To help summarise all the information gathered during 2DE, the presence or absence of proteins in each species were tabulated into two matrices. These allowed the data produced

from 2DE to be analysed using two parsimony programmes, each of which made different assumptions about the rate of accumulation of mutations (see Section 2.2.8). While both methods were in agreement within the two tissues examined, the protein data from guts and remnants did not produce the same relationships between species, (Section 3.2.2). The two tissue types showed differences in the numbers and types of proteins present and in the level of variation seen between species and only the gut data produced strong relationships after parsimonious analysis. Because the species trees produced were based on the presence or absence of a protein (a "+" or a "-") the data could not be used to establish branch lengths based on the accumulation of point mutational differences

Based on the information produced after dendrogram analysis, it would appear that the gut protein data generated are compatible with the established morphological methods of separating aphid species (Heie, 1980). At the species level, future 2DE comparisons of gut or remnant protein profiles may help in the recognition of different aphid species where morphological information is lacking. Where morphologically indistinguishable aphids are thought to exist in species complexes, gut protein profiles may be useful in disclosing whether more than one species is present. For example, *M. persicae* and *M. antirrhinii* are very closely related species which are difficult to distinguish morphologically and their identification requires the use of both morphometrics and karyotyping (Blackman and Paterson, 1986). However, in the current work, a comparison of the gut proteins from these two species showed differences which would allow them to be separated (Table 3.3). It is possible that further differences could be established by probing the gut proteins with anti-*M. persicae* gut antibodies or other probes to detect distinguishing proteins (Figure 5.3).

The work described in this thesis focused on the proteins found in guts and remnants, and the way in which these varied in comparison to each other and between species. However, it is possible that the protein profiles of other individual aphid tissues varied as much as those found in guts, with the various tissues represented in the remnants contributing different proportions of varying and conserved proteins to the observed remnant patterns. To investigate this further would require isolation of sufficient amounts of individual tissues from remnants to allow analysis of their proteins after 2DE separation. Bearing in mind that a lot of any aphid is composed of gut tissue, reproductive tract and exoskeleton (Miyazaki, 1987; Ponsen, 1987), the isolation of any other individual tissue, such as the fat bodies, would be extremely time consuming and is probably not a practical proposition. Changes to the IEF method employed for 2DE could also reveal other proteins already present in guts and remnants. For example, expanding the pII range by increasing the range of ampholines used during the IEF phase of 2DE (see Section 2.2.7.2) would help to resolve those proteins with pIs outside the range examined. A few proteins can be seen on 2DE as smears at the origin of the IEF gels. However, the number of proteins resolved with the current pII range of 2DE (Tables 3.1 and 3.3; >235 in each species) compares very favourably with the numbers resolved by 1DE (Figure 3.1; > 90). The expansion of the present IEF pH range would therefore be expected to yield few extra proteins.

## **7.2 Induced Differences In Aphid Gut Proteins**

Aside from the protein variation already described between aphid species, work described in Chapter 4 found that alterations to aphid diet caused changes in the gut

proteins found within a species. After the possible influence of genetic variation within species had been eliminated with the use of clones, the same differences in gut protein profile associated with feeding on distinct host plants were still found.

Changes in gut proteins have been seen in other insects in response to alteration to their diet. For example, when plant PIs were added to the diet of herbivorous insects changes occurred in digestive proteases (Broadway, 1989, 1996 a). These changes included up regulation of existing enzymes which appeared to be sensitive to the plant compounds and the *de novo* synthesis of PI-insensitive enzymes. A recent investigation of changes in gene expression of digestive proteinases in Lepidoptera found differential regulation of the proteinases in response to inclusion of plant PIs in the diet (Gatehouse *et al.*, 1997). While the regulatory mechanisms responsible for this differential secretion of digestive enzymes are not known there is speculation that hormonal factors may be involved (Broadway, 1995).

While the differences in gut proteins when *M. persicae* was switched between host plants consisted of only a small proportion of the total gut protein profile (see Section 4.2.4), the variation observed represented protein production being turned both on and off. The changes may represent a gut control mechanism which responds to dietary components with the production of proteins useful to processing the new diet or which protect the gut from harmful ingested substances. A feedback mechanism which reacts to the presence of factors in the diet, such as the induction of alcohol dehydrogenase in the liver with the presence of alcohol (Alberts *et al.*, 1989), could produce the on/off effects seen. An example of this type of mechanism could be the gradual reduction of esterase production in some insecticide resistant *M. persicae* clones when insecticide selection is absent, and the restoration of elevated esterase activity

when selection is reapplied (Ilick *et al.*, 1996). Cessation of production of redundant proteins in favour of useful ones would also be an efficient use of resources in aphids, which exist on a very tight nutritional budget (Srivastava, 1987). A crucial test would be to see if the proteins apparently expressed after feeding on a new host plant eventually disappeared following movement of the aphids back to the original host, and concomitantly whether the original protein pattern was restored.

In this thesis, the examination of the changes induced in aphid gut proteins by dietary factors used clonally derived aphids. As has been mentioned before (Section 4.2.1), the use of these genetically identical individuals to analyse the observed protein variation has allowed the elimination of genotypic differences as a causal factor. In the same way, the use of aphid clones would be of great benefit when exploring the mechanisms behind the variation in gut protein using such methods as differential display (Liang and Pardee, 1992). This technique would help to identify the RNA expressed from genes which are being turned on and off as the aphids adapt to a new host plant. Identification of differentially regulated genes could ultimately lead to an understanding of the mechanism controlling this process. Another positive element for future investigation of the aphid gut protein control mechanism by differential display is that the number of proteins varying between plants as a subset of the total number of gut protein is relatively small and will allow further research to be focused immediately on identified targets.

Another variation that can occur within clones is the production of different types of offspring. Many aphids are able to alter the phenotype of progeny produced (alatae, apterae, males, etc.) and the production of these different physical and sexual morphs may cause further changes in the proteins present in the aphid. Because of this

ability, the physical structure of the aphid reproductive tract has been examined in great detail. However, for reasons already explained (Section 2.2.1.1) most of the proteins within the reproductive tract were excluded from study in this thesis, leaving the protein profile of this major system unexamined. The reproductive tract was the only major aphid tissue that was not examined. As aphids can be artificially stimulated to produce different forms of progeny by external factors, such as crowding (alate production) or changes to dietary components and daylength (males or gynoparae), it would be possible to investigate changes in the proteins of the reproductive tract as switches in progeny type were induced. It is possible that such an examination could reveal key proteins involved in the aphid reproductive and dispersal strategies, providing not only information on basic aphid biology, but also offering new targets for their control.

### **7.3 Implications Of The Variation In Aphid Gut Protein**

Having found high levels of protein variation between the guts of different aphid species it will now be important to establish what factors are causing this phenomenon. Each species has a complement of gut proteins which should logically be involved in processing food and in protecting the gut against damage caused by ingested material. Because the host ranges of very few aphids coincide exactly, the gut proteins have to respond to the different diets of each species and within species, the alterations to the gut protein profile appear to be part of a specific response to dietary changes. Research carried out on other insects found that polyphagous species were more likely to be able to feed successfully in the presence of plant PIs from plants outside their host range than monophagous species (Broadway and Villani, 1995). The ability to feed on different



host plants containing a variety of plant PIs appeared to have pre-adapted these polyphagous insects to either be insensitive to PIs similar to host-PIs or to overcome the deleterious effects of these PIs with changes in protein production (Broadway, 1996 b; Jongsma and Boulter, 1997). Many of the putative plant defence chemicals are normally only present in storage organs or vegetative tissues (Felton and Gatehouse, 1996), with very low levels found in the phloem (Douglas, 1993) and it therefore seems unlikely that these compounds are encouraging the production of different gut proteins in aphids as they feed exclusively on phloem sap. However, even very low natural concentrations of plant chemicals, with no apparent deleterious effect on the aphid, may cause a response in the aphid gut. This, in turn, can enable the aphids to feed in the presence of higher concentrations of the compound on different plants or other crop cultivars containing higher levels of the compound. For example, after *M. persicae* were fed on lupins with low levels of alkaloids in the phloem, they were able to feed on other cultivars of lupin with much higher alkaloids concentrations that would normally have been deleterious to the aphid (Berlandier, 1996). Furthermore, a wide range of aphids are known to be able to feed successfully in the presence of a wide range of lectin concentrations (Rahbé and Febvay, 1992; Rahbé *et al.*, 1995). The physiological concentration of most lectins within phloem sap is between 20 and 200  $\mu\text{g ml}^{-1}$ , while there are occasional exceptions such as the Cucurbitaceae which can have lectin concentrations of up to 1  $\text{mg ml}^{-1}$ , and aphids were capable of feeding in the presence of these physiological lectin concentrations (Rahbé and Febvay, 1993). Aphids have been shown to produce new proteins in response to host alteration (Egaas *et al.*, 1991), possibly as a direct consequence of contact with novel plant compounds. If this process

was repeated with each plant compound encountered, a large variety of proteins would be required in the aphid gut to allow successful feeding.

The observed changes induced in aphid gut proteins by dietary factors may have some cost to the aphid. The evidence from feeding trials found that longevity and fecundity were reduced after aphids were transferred to different host plants (Owusu *et al.*, 1996). However, aphids have been found to eventually adapt to new dietary environments, with a restoration of previous levels of reproduction, etc. (Douglas, 1997; Wool *et al.*, 1995). Therefore, the initial costs to expansion of aphid populations onto different crops each with a distinct phloem composition, would be recouped when the aphids were able to exploit the novel host plant. In natural circumstances the adaptation of insects to plant defence compounds is just one side of a balanced polymorphism maintained between plant and pest. Where the plant evolves anti-pest mechanisms, the pests evolve to overcome these barriers, leading to subtle and probably continuous changes in the plant-pest relationship. When this balance is disturbed, for example through limited selection of genotypes for crops, the pests start to have an advantage. Many pest insects go through several generations per year and with each generation there is the chance to accumulate favourable mutations or increase the proportions of a favourable genotype within a population. However, this process would be slower in aphids, as although they can produce many generations in a year most of these are parthenogenetic and therefore there is no chance for favourable recombination to occur (Hales *et al.*, 1997). Once released onto commercial markets, crop varieties have no chance to produce beneficial mutations against pests, with only one generation per year, at most, and they are selected for their genetic uniformity. In the case of aphids, it would appear that genotype alterations may not be necessary for exploitation of new

host plants. Aphid clones are produced without any genotype recombination taking place (Hales *et al.*, 1997) and the work in this thesis has shown that such genetically identical clones are able to successfully use several host plants.

The inter-specific variation in aphid gut proteins also has a number of implications for the current trend of plant based aphid control. When transgenically imported plant factors aimed at the gut are incorporated into the aphid diet, the current evidence would suggest that aphids, and particularly polyphagous aphids, may be pre-programmed for rapid adaptation to them. In addition, having observed the range of protein variation between and within species, there is no reason to assume the response to any novel control mechanism would be the same across all aphid species. Therefore, any strategy which has the aphid gut as a target, such as the expression of specific lectins in crop plants (e.g. Down *et al.*, 1996), would have to take the levels of inter-specific variation into account in order to be effective in the field. As there is frequently more than one species of aphid found feeding on any single crop, multiple resistance mechanisms would be required to achieve effective protection against all aphid species. Not only will this increase the costs of developing new crop varieties, but the very control mechanisms used would increase pressure on the aphids to adapt. Coupled with a lack of knowledge as to the exact way in which many of these proposed control methods actually work in the plants (McManus *et al.*, 1994; Czapla, 1997), there is a potential for creating more problems with insect resistance.

#### **7.4 A Novel Target For Aphid Control**

During the work carried out for this thesis, it was observed that the inclusion of

anti- *M. persicae* gut antibodies in AD had a deleterious effect on feeding aphids. Antibodies raised against aphid gut proteins appeared to reduce the longevity of aphids in comparison to AD (see Section 6.3) and there are methods which could now be used to overcome the confounding factors that previously interfered with any statistical proof of effect. These methods were discussed in Chapter 6. However, the results of the antibody feeding trials do indicate that antibodies or functional antibody fragments, specifically active against aphid antigens, could form the basis of a method of aphid control. This type of technique would mirror that used to produce protective immunisation against animal parasites, but for aphids the delivery of the control factors would require transgenic plants with the antibody expressed in the phloem.

When the antibodies raised against aphid proteins were characterised, interesting information about the nature of many aphid gut proteins was revealed. The examination of antibody cross reactivity confirmed the presence of proteins which were conserved between aphid species and, in conjunction with lectins, which bind specifically to glycoproteins, high levels of glycosylated proteins in the aphid gut. As the level of gut protein conservation did not appear to be very high after analysis by 2DE, this may have indicated that some of the proteins occurring in different species were in fact modified homologues of proteins common to all species. If this is the case, it would suggest that the amino acid sequence of the proteins have common regions, perhaps in and around active sites, therefore control of different aphid species would be ideally suited to an anti-aphid antigen approach. Ideally, a single essential protein with homologues in other aphid species could be used as the antigen with the subsequent antibody product binding to the active site of the antigen, affording protection against all the aphids with homologues.

However, there may be another explanation for the level of cross reactivity seen. In investigations of the potential protective effects of anti-parasite antibodies raised against proteins from one parasite species, cross reactivity with proteins from related species have sometimes been found (e.g. McTier *et al.*, 1981). The cross reactivity did not always afford protection against the related parasites and the antibodies were sometimes found to be reacting with protein secondary structures, such as glycosylated areas, in these cases (Xu and Powell, 1991). The cross-reactivity could be removed by deglycosylation of proteins prior to probing with the antibody (Xu and Powell, 1991). In parallel with this, deglycosylation of antigens from the tick *B. microplus* before immunisation was found to remove the protective effect against the same tick (Lee *et al.*, 1991). As mentioned above, the anti-gut antibody was found to react with a large number of glycosylated proteins in the aphid gut. Studies involving deglycosylation of the proteins to be probed would have to be undertaken to assess whether this would reduce binding to the guts of the donor aphid, *M. persicae*, and if cross-reaction with proteins from other aphids was prevented. It should be noted that not all of the proteins recognised by the antibodies were glycosylated, and therefore the relative importance of gut protein glycosylation, in both the normal functions of the aphid gut and in adaptation to dietary factors, is still not known.

Of equal importance to identifying the reasons for cross reactivity is an assessment of whether this can be translated into a deleterious effect of the antibody on other species of aphid. So far, feeding trials have only tested the anti-gut antibody against *M. persicae*. The limiting factor in such investigations with other aphid species is finding an AD to support them. During the course of this research attempts were made to maintain *Ma. euphorbiae*, *A. idaei* and *M. antirrhinii* on AD, with limited

success. However, if subdivision of the current anti-gut antibody, for example to produce Fab fragments, were to produce a rapid adverse effect on the aphids under test, it would not be necessary to find chemically defined diets to keep the various species in long term culture. Further fractionation of the aphid gut antigens used as immunogen, for example with lectins of different binding specificity, could also yield improved aphicidal antiserum (see Section 6.4).

The aphid gut has proved to contain many potential protein targets which could be manipulated to produce aphid control. The mechanisms underlying the observed protein variation may also prove to be useful aphid control targets, where changes in these mechanisms could be specifically induced in the target aphids. However, the variation observed in the gut tissue has also highlighted the need for careful choice of targets, based on accurate protein characterisation and clear objectives for control. For example, where the objective is to prevent transmission of plant viruses at a time of critical plant growth, it may be possible to have developmentally or externally regulated expression of aphid control factors in the plant. Recent research has produced plants which can be induced to express a transgene after external application of ethanol (Caddick *et al.*, 1998). The use of such inducible gene expression may allow the development of plant-based aphid control mechanisms which can be activated as required without causing a continuous burden to the plant. Induced expression of plant defensive factors may also produce effective aphid control while not continuously exposing the aphids to a selective pressure, thereby reducing the probability of aphid adaptation to the control factor.

## 7.5 Conclusions

The work carried out for this thesis has characterised over 230 proteins in aphid tissues and has analysed the variation in gut and remnant protein profiles of six aphid species. Changes were observed within the gut protein profiles of two aphids species after they were moved between two unrelated host plants, with absences and additions to the protein profiles. Antibodies, raised against gut proteins from *M. persicae* fed on Chinese cabbage, recognised the additional proteins present in gut tissue when *M. persicae* fed on potato and also many of the proteins found in the guts of other aphid species. Inclusion of these antibodies in AD appeared to lead to a reduction in longevity of aphids feeding on this mixture, although this was not statistically significant.

The variation seen between the gut proteins of the aphid species examined, and the changes observed in these proteins within a species, after alteration of diet, have provided novel information on the basic biology of aphids. From the protein variation seen in both of these situations, it is likely that future aphid control strategies which generally target the aphid gut will produce changes in the protein constituents of the aphids.

Basic information on the quantities and types of aphid gut protein, and the genetic control underlying their production, may be very useful for the development of effective strategies to engineer complementary plant resistance mechanisms to these pests. The studies carried out for this thesis have established the protein background against which future changes can be measured, allowing further investigations of protein variation within aphid species to proceed with greater speed and focus.

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## **Publications and Abstracts of Scientific Meetings**

### **Publications**

Irving, P. and Fenton, B. (1995) Molecular and adaptive variation in aphid guts.  
*SCRI Ann Rep* 154-155

Irving, P., Stanley, K., and Fenton, B. Inter-specific variation in aphid gut proteins detected by two-dimensional electrophoresis. (in prep.)

Irving, P. and Fenton, B. Analysis of within clone variation in aphid gut proteins. (in prep.)

### **Presentations**

A molecular analysis of aphid gut proteins.  
(February 1996) Scottish Molecular Ecology Group Meeting, Dundee

A 2DE comparison of aphid gut proteins.  
(September 1997) Abstracts of Entomology '97, Royal Entomological Society, University of Newcastle, Newcastle-upon-Tyne.

### **Posters**

- (1) A molecular analysis of aphid guts.
- (2) Is there a peritrophic-like membrane in aphid guts?  
(October, 1995) Aphid Special Interest Group. Royal Entomological Society Meeting, London

## Appendix 1

### Raw data for Figure 6.1

Adult *M. persicae* were loaded onto diet sachets as follows:- three AD sachets containing NSS (1:250), each loaded with nine *M. persicae*; three AD sachets containing NSS (1:500), each loaded with eight *M. persicae*; three AD sachets containing anti- whole *M. persicae* antibody (1:250), each loaded with nine *M. persicae*; three AD sachets containing anti- whole *M. persicae* antibody (1:500), each loaded with nine *M. persicae*. Results of the feeding trial are given as total numbers (and percentage) of adults surviving.

Adult survival (%)	1:250 NSS	1:500 NSS	1:250 $\alpha$ whole <i>M.p.</i>	1:500 $\alpha$ whole <i>M.p.</i>
day 1	27 (100)	24 (100)	27(100)	27 (100)
day 2	27 (100)	24 (100)	27 (100)	27 (100)
day 3	27 (100)	18 (75)	21 (77)	20 (75)
day 4	9 (100)	12 (50)	14 (52)	10 (37)
day 5	0 (0)	6 (25)	0 (0)	10 (37)
day 6	0 (0)	3 (12.5)	0 (0)	0 (0)

### Raw data for Figure 6.2

Adult *M. persicae* were loaded onto diet sachets as follows:- two AD sachets each loaded with ten *M. persicae*; two AD sachets containing NSS (1:50), each loaded with ten *M. persicae*; two AD sachets containing NSS (1:200), each loaded with ten *M. persicae*; two AD sachets containing NSS (1:500), each loaded with ten *M. persicae*; two AD sachets containing anti- *M. persicae* gut antibody (1:50), each loaded with ten *M. persicae*; two AD sachets containing anti- *M. persicae* gut antibody (1:200), each loaded with ten *M. persicae*; two AD sachets containing anti- *M. persicae* gut antibody (1:500), each loaded with ten *M. persicae*. Results of the feeding trial are given as total numbers (and percentage) of adults surviving.

Adult Survival (%)	AD	NSS 1:50	NSS 1:200	NSS 1:500
day 0	20 (100)	20 (100)	20 (100)	20 (100)
day 1	20 (100)	20 (100)	20 (100)	20 (100)
day 2	20 (100)	20 (100)	19 (95)	18 (90)
day 3	19 (95)	18 (90)	16 (80)	16 (80)
day 4	17 (85)	18 (90)	16 (80)	15 (75)
day 5	17 (85)	18 (90)	16 (80)	15 (75)
day 6	17 (85)	16 (80)	15 (75)	13 (65)

Adult Survival (%)	Gut Ab 1:50	Gut Ab 1:200	Gut Ab 1:500
day 0	20 (100)	20 (100)	20 (100)
day 1	19 (95)	20 (100)	20 (100)
day 2	17 (85)	19 (95)	17 (85)
day 3	12 (60)	17 (85)	14 (70)
day 4	6 (30)	17 (85)	13 (65)
day 5	6 (30)	17 (85)	13 (65)
day 6	4 (20)	8 (40)	7 (35)

### Raw data for Figure 6.3

Adult *M. persicae* were loaded onto diet sachets as follows:- five AD sachets each loaded with fifteen *M. persicae*; five AD sachets containing NSS (1:50), each loaded with fifteen *M. persicae*; five AD sachets containing anti- *M. persicae* gut antibody (1:50), each loaded with fifteen *M. persicae*. Results of the feeding trial are given as total numbers (and percentage) of adults surviving.

Treatment	day 0	day 1	day 3	day 5	day 7	day 9	day 12
Antigut antiserum	75 (100)	66 (88)	64 (85)	58 (77)	45 (61)	37 (50)	14 (19)
NSS	75 (100)	75 (100)	71 (95)	68 (91)	55 (73)	47 (63)	28 (38)
AD	75 (100)	74 (99)	69 (92)	68 (91)	61 (81)	55 (73)	42 (56)

### Raw data for Figure 6.4

Adult *M. persicae* were loaded onto diet sachets as follows:- four AD sachets each loaded with fifteen *M. persicae* and one with fourteen; five AD sachets containing NSS (1:50), each loaded with fifteen *M. persicae*; five AD sachets containing anti- *M. persicae* gut antibody (1:50), each loaded with fifteen *M. persicae*. Results of the feeding trial are given as numbers (and percentage) of adults surviving on each diet sachet.

#### 1. Adult aphid survival on AD containing anti- *M. persicae* gut antiserum (1:50)

##### Adult survival - absolute numbers (and % survival)

Sachet number	day 0	day 1	day 3	day 5	day 7	day 9	day 12
1A	14 (100)	14 (100)	13 (93)	13 (93)	12 (86)	10 (71)	6 (43)
1B	15 (100)	15 (100)	15 (100)	15 (100)	14 (93)	11 (73)	0 (0)
1C	15 (100)	13 (87)	13 (87)	9 (60)	0 (0)	0 (0)	0 (0)
1D	15 (100)	9 (60)	8 (53)	8 (53)	8 (53)	5 (33)	2 (13)
1E	15 (100)	14 (93)	14 (93)	13 (87)	12 (80)	11 (73)	6 (40)

## 2. Adult aphid survival on AD containing NSS (1:50)

Adult survival - absolute numbers (and % survival)

Sachet type & no.	day 0	day 1	day 3	day 5	day 7	day 9	day 12
2A	15 (100)	15 (100)	15 (100)	15 (100)	12 (80)	9 (60)	8 (53)
2B	15 (100)	15 (100)	15 (100)	14 (93)	14 (93)	14 (93)	13 (87)
2C	15 (100)	15 (100)	13 (87)	12 (80)	8 (53)	7 (47)	4 (27)
2D	15 (100)	15 (100)	14 (93)	14 (93)	10 (67)	9 (60)	3 (20)
2E	15 (100)	15 (100)	14 (93)	13 (87)	11 (73)	8 (53)	0 (0)

## 3. Adult aphid survival on AD

Adult survival - absolute numbers (and % survival)

Sachet type & no.	day 0	day 1	day 3	day 5	day 7	day 9	day 12
3A	15 (100)	15 (100)	15 (100)	14 (93)	14 (93)	12 (80)	9 (60)
3B	15 (100)	15 (100)	13 (87)	13 (87)	12 (80)	11 (73)	11 (73)
3C	15 (100)	15 (100)	13 (87)	13 (87)	13 (87)	13 (87)	10 (67)
3D	15 (100)	15 (100)	14 (93)	14 (93)	11 (73)	10 (67)	7 (47)
3E	15 (100)	14 (93)	14 (93)	14 (93)	11 (73)	9 (60)	5 (33)

The data were in the form of repeated measures, where each individual aphid was not distinguished within the data sets. The percentage of adults surviving over 12 days was monitored for three types of diet regime. Five replicates (sachets) were used for each treatment, with 14 or 15 adults in each replicate. Figure 6.4 and associated data display the results, suggesting that percentage survival falls over time.

An approximate statistical test for the comparison of AD including anti-gut antibody versus NSS (based on the results for day 9 or 12) did not, however, prove to be statistically significant, using a non-parametric test [Mann-Whitney two-sample rank tests on the percentage survival data gave  $p = 0.4034$  of the two test diets giving different survival rates at day 12, and on day 7 the probability reduced to  $p = 1.0000$ ].

